REMARKS

The Present Invention

The present invention pertains to a lymphocyte having dual specificity, compositions comprising the same, a pharmaceutical composition comprising the same, and a method of preparing the same.

The Pending Claims

Claims 1, 4, 7, 8, 10, 11, 40, 41, 44-61, and 72-82 are pending. Claims 1, 4, 7, 8, 10, 46, and 72-78 are directed to a composition comprising a dual specificity T lymphocyte. Claims 11 and 47-51 are directed to a dual specificity lymphocyte. Claims 40 and 52-56 are directed to a pharmaceutical composition comprising a dual specificity T lymphocyte. Claims 41 and 58-61 are directed to a method of preparing lymphocytes having dual specificity, while claim 71 is directed to the lymphocytes made therefrom. Claims 79-82 are directed to a composition comprising a population of T lymphocytes.

The Office Action

The Office Action objects to claims 1, 11, and 40 for the recitation of the phrase "a cell, which cell is" and suggests to amend the claims to instead recite "a cell that is." The Office Action maintains the rejection of claims 1, 3, 4, 7, 8, 10, 11, 40, 41, and 44-61 and rejects claim 71 under 35 U.S.C. § 112, first paragraph, as allegedly containing new matter. The Office Action further maintains the rejection of claims 1, 3, 4, 7, 8, 10, 11, 40, 41, and 44-61 and rejects claim 71 under 35 U.S.C. § 112, first paragraph, as allegedly lacking written description. The Office Action maintains the rejection of claims 1, 3, 4, 7, 8, 10, 11, 40, 41, and 44-61 and rejects claim 71 under Section 112, second paragraph, as allegedly indefinite. Furthermore, the Office Action maintains the rejection of claims 1, 3, 4, 7, 8, 10, 11, 40, 41, and 44-61 and rejects claim 71 under 35 U.S.C. § 102 (e) as being anticipated by U.S. Patent 5,830,755. Also, claims 1, 3, 7, 8, 11, 40, 41, 45-47, 50, 52, 56, 58, and 61 remain rejected under Section 102 (e) as allegedly anticipated by U.S. Patent 6,407,221. Claims 1, 3, 7, 8, 11, 40, 41, 45-67, 50, 52, 56, 58, and 61 remain rejected and claim 71 is rejected under Section 102 (e) as allegedly anticipated by U.S. Patent 5,359,046. Reconsideration of the objection and rejections is hereby requested.

Discussion of the Amendments to the Claims

The claims have been amended to place them in better form for appeal. For example, pending claim 1 has been amended to remove certain terms which are recited in new claims 72-76. As noted below, this amendment obviates the concern expressed in the Office Action that claim 1 may be unclear because these claim terms are expressed together in claim 1. Additionally, the Office Action alleges that the claims may be inherently anticipated by the prior art. New claims 77-82 are directed to subject matter, which applicants believe cannot be inherently anticipated by the prior art. Moreover, the claims have also been amended as explicitly suggested by the Office Action. Accordingly, applicants respectfully submit that the claim amendments should be entered under 37 C.F.R. § 1.116.

Specifically, claims 1, 11, 40, and 41 have been amended to recite "a cell that is allogeneic," as suggested by the Office. Claim 1 has been further amended to delete "recombinant T-cell receptor, either of" from part (i). This subject matter is the subject of new claims 72-76. Claims 77-82 have also been added, which are supported in the specification by, for instance, page 31, line 4, of paragraph 81 through page 32, line 14, of paragraph 81. Claims 80 and 82 are further supported by paragraph 0053 on page 17. Accordingly, no new matter has been added by way of the amendments.

Discussion of the New Matter Rejections

The Office Action maintains the rejection of claims 1, 3, 4, 7, 8, 10, 11, 40, 41, and 44-61 and rejects claim 71 under 35 U.S.C. § 112, first paragraph, as allegedly containing new matter. This rejection is traversed for the reasons set forth below.

The Office contends that the scope of lymphocytes having a second receptor that recognizes any cell that is allogeneic to the lymphocyte is new matter. However, such T lymphocytes are supported by the instant specification at, for example, page 6, paragraph 17, and page 11, lines 1-12 of paragraph 41, which discloses that the antigen to which the second receptor reacts includes any strong antigen, such as an allogeneic cell. Moreover, originally filed claims 1, 2, 12, 13, and 15 cannot be rejected as "new matter" because they are original claims, yet the allegation in the Office Action would encompass these claims. While other support for these claims can be found in the application, this teaching of the instant specification alone is enough to demonstrate that the subject matter is not new.

The Office apparently maintains the new matter rejection because Examples 3-9 and 11, which were previously cited by Applicants, allegedly only teach stimulating the cells with PBMC and determining if the cells recognize allogeneic PBMC. Example 5 discloses dual specificity T cells having a receptor which is reactive to allogeneic splenocytes (page 30, line

3, of paragraph 79) and a chimeric receptor reactive to a tumor antigen, Folate Binding Protein (FBP) (page 30, line 2 of paragraph 79). Example 9 discloses cells having a receptor which is reactive to one of allogeneic PBMC, B cells, and dendritic cells (page 35, line 8, of paragraph 83) and a chimeric receptor (Mov-γ) (page 35, line 11 of paragraph 83). In this regard, PBMCs are not the only allogeneic cells that are used to stimulate the claimed dual specific T cells. Accordingly, the rejected claims do not comprise new matter, nor does the instant specification fail to disclose T cells having a T cell receptor reactive to allogeneic cells other than allogeneic PBMC.

The Office also alleges that the specification does not teach that the receptor will recognize *any* allogeneic cell as broadly claimed. Originally filed claims 1 and 2 recite the subject matter objected to. Originally filed claims are part of Applicants disclosure. Accordingly, this subject matter cannot be "new."

The Office further alleges that the T lymphocyte having two receptors comprising an MOv-γ receptor and an endogenous receptor that reacts with a splenocyte, dendritic cell, B-cell or peripheral blood cell that is allogeneic to the lymphocyte is new matter. As described above, the Examples demonstrate T cells having an endogenous receptor reactive to one of allogeneic splenocytes, dendritic cells, B cells, and PBMCs.

The Office contends that the specification does not teach that the "dual specificity allogeneic/MOv-γ T cells" had an endogenous T cell receptor reactive with splenocytes. When a T cell receptor reacts to the antigen to which it binds, a number of T cell responses can occur, one of which is T cell proliferation, i.e., clonal expansion. See Janeway et al., Immunobiology, 5th ed., Garland Publishing, New York, NY, 2001, pages 20-21. Figures 3A and 3B of the instant application demonstrate that the number of Thy 1.1 T cells was remarkably increased when the recipient mice received an immunization with splenocytes (black bars), in contrast to the mice that did not receive the splenocyte immunization (white bars). This experiment demonstrates that the splenocytes caused the Thy 1.1 T cells to clonally expand, i.e., to proliferate, which occurred due to the endogenous T cell receptor reacting to the splenocytes. Thus, the ordinarily skilled artisan would readily and immediately understand that the instant application does support dual specificity T cells having an endogenous T cell receptor reactive with allogeneic splenocytes.

In view of the foregoing, the instant claims do not contain new matter. Therefore, Applicants respectfully request that the new matter rejection of claims 1, 3, 4, 7, 8, 10, 11, 40, 41, 44-61, and 71 be withdrawn.

Discussion of the Written Description Rejection

The Office Action maintains the rejection of claims 1, 3, 4, 7, 8, 10, 11, 40, 41, and 44-61 and rejects claim 71 under 35 U.S.C. § 112, first paragraph, as allegedly lacking adequate written description. Specifically, the Office contends that the "chimeric receptor reactive with a tumor antigen" does not meet the written description requirements, because the instant specification does not disclose the alleged required DNA sequences of all the chimeric receptors encompassed within the scope of the claim. This rejection is traversed for the reason set forth below.

First, the ordinarily skilled artisan would, upon reading the specification, conclude that Applicants were in possession of the claimed invention. At least for this reason, the application contains an ample written description of the claimed invention.

Second, as noted by the Manual of Patent Examining Procedure (MPEP) (§ 2163), the description need only describe in detail that which is new or not conventional. See *Hybritech v. Monoclonal Antibodies*, 802 F.2d at 1384, 231 USPQ at 94; *Fonar Corp. v. General Electric, Co.*, 107 F.3d at 1549, 41 USPQ2d at 1805. In the instant case, chimeric receptors reactive with a tumor antigen were known in the art at the time of filing the subject patent application. For example, a chimeric receptor reactive to a tumor antigen expressed in colon carcinoma is described in Haynes et al., *J Immunol* (January 2001) 166: 182-187 (article attached hereto). Also, a chimeric receptor reactive to a tumor antigen expressed in breast cancer is described in Dakappagari et al., *Cancer Res* (July 15, 2000) 60: 3782-3789 (article attached hereto), and chimeric receptors reactive to a renal cell carcinoma antigen are described in Weijtens et al., *J Immunol* (July 15, 1996) 157: 836-843 (article attached hereto), Weijtens et al., *Gene Ther* (September 1998) 5: 1195-1203 (abstract attached hereto), and Weijtens et al. *Int J Cancer* (July 17, 1998) 77: 181-187 (abstract attached hereto). Accordingly, because such chimeric receptors were known in the art at the time of filing the instant application, the specification is not required to include DNA sequences of the chimeric receptors.

In view of the foregoing, claims 1, 3, 4, 7, 8, 10, 11, 40, 41, 44-61 and 71 are adequately described. Therefore, Applicants request that the rejection for lack of written description be withdrawn.

Discussion of the Indefiniteness Rejection

The Office Action maintains the rejection of claims 1, 3, 4, 7, 8, 10, 11, 40, 41, and 44-61 and rejects claim 71 under Section 112, second paragraph, as allegedly indefinite. Specifically, the Office alleges that the phrase "recombinant chimeric receptor" or

"recombinant T cell receptor" in the context of claim 1 does not make sense. Applicants thank the Examiner for helping to ensure that the pending claims are clear. To obviate the rejection, applicants have separated the claim terms at issue into separate claims. In view of the amendments to the claims (i.e., cancellation of "recombinant T cell receptor" from claim 1, and the addition of new claim 72) this rejection does not apply to the claims as pending.

Discussion of the Anticipation Rejections

The Office Action maintains the rejection of claims 1, 3, 4, 7, 8, 10, 11, 40, 41, and 44-61 and rejects claim 71 under 35 U.S.C. § 102 (e) as being anticipated by U.S. Patent 5,830,755 (the '755 patent). Also, claims 1, 3, 7, 8, 11, 40, 41, 45-47, 50, 52, 56, 58, and 61 remain rejected under Section 102 (e) as allegedly anticipated by U.S. Patent 6,407,221 (the '221 patent). Claims 1, 3, 7, 8, 11, 40, 41, 45-67, 50, 52, 56, 58, and 61 remain rejected and claim 71 is rejected under Section 102 (e) as allegedly anticipated by U.S. Patent 5, 359,046 (the '046 patent). These rejections are traversed for the reasons set forth below.

The Office interprets the '755 patent in three different ways. In a first interpretation, the TIL of the '755 patent purportedly inherently have an endogenous T cell receptor reactive with a cell that is allogeneic to the T cell because both transduced and non-transduced TIL reacted with murine sarcoma cells (24JK) (Table 8). However, as stated in the Declaration of Dr. Patrick Hwu, the 24JK cells are not allogeneic to the TIL. Rather, the cells are syngeneic to each other. Accordingly, the '755 patent when viewed according to the Office's first interpretation does not anticipate the instant claims.

In a second interpretation of the '755 patent, the Office contends that the endogenous T cell receptor that reacts to a cell that is allogeneic to the T cell is inherent to the TIL disclosed in the '755 patent, since the population of TIL was diverse. The Office speculates that one of the many and diverse T cell receptors in a population of TIL should recognize an allogeneic cell. This speculation, however, is not supported by evidence. Under the principles of inherency, the prior art must necessarily function in accordance with the claims it allegedly anticipates. *Atlas Powder Co. v. Ireco, Inc.*, 190 F.3d 1342 (Fed. Cir. 1999); *In re King*, 801 F.2d 1324 (Fed. Cir. 1986). Inherency may not be established by probabilities or possibilities. The mere fact that a certain thing *may* result from a given set of circumstances is not sufficient. *Continental Can Co. USA, Inc. v. Monsanto Co.*, 948 F.2d 1264 (Fed. Cir. 1991) (emphasis in original). In the instant case, there is no evidence of record that demonstrates that the diversity of the TIL population guarantees that the population contains a T cell having an endogenous T cell receptor reactive to an allogeneic cell. Accordingly,

inherency cannot be demonstrated in the instant case. Therefore, the '755 patent does not anticipate the claimed invention under the Office's second interpretation.

In a third interpretation, the Office alleges that the '755 patent discloses TIL which are exposed to an antigen prior to transduction to stimulate growth and expansion of cells that recognize the antigen. However, the '755 patent does not disclose that the antigen is an allogeneic cell. Accordingly, the '755 patent does not anticipate the claimed invention under the Office's third interpretation.

The Office also rejects claim 41 as allegedly anticipated by the '755 patent, since the steps of the method of claim 41 are allegedly disclosed by the '755 patent. However, as stated above, the '755 patent does not disclose contacting lymphocytes with a cell that is allogeneic to the lymphocytes. Thus, the '755 patent does not anticipate the method of claim 41.

In view of the foregoing, the '755 patent does not anticipate the instantly claimed invention. Therefore, Applicants respectfully request that the rejection under § 102 in view of the '755 patent be withdrawn.

The Office Action alleges that the '221 and '046 patents anticipate claims 1, 3, 7, 8, 11, 40, 41, 45-47, 50, 52, 56, 58, 61, and 71. Specifically, the Office Action alleges that the '221 and '046 patents disclose T cells transduced with a vector encoding a chimeric receptor that recognizes a tumor antigen. In some instances, the tumor antigen allegedly is the HIV protein gp120. The Office further alleges that the T cells of the '221 and '046 patents inherently comprise an endogenous T cell receptor, which is reactive to an allogeneic cell, since the T cells were in a diverse population of T cells. The rejection of the claims under § 102 in view of the '221 patent is traversed for the reasons set forth below.

The Office alleges that the HIV gp120 protein is considered as a tumor antigen because the protein was caused to be expressed in a tumor cell. However, "tumor antigen" is defined in the instant application on page 13, lines 8-15 of paragraph 0046, as a molecule that can be used to target therapy against a tumor and includes those antigens only found on tumor cells (i.e., tumor specific), those which are expressed on tumor cells and on limited normal tissues, and those which are over-expressed on tumor cells compared to the expression on a wide variety of normal tissues (i.e. over-expressed antigens). At least because the gp120 protein is not a molecule that can be used to target therapy against a tumor, this protein cannot be considered as a tumor antigen as defined by the instant application.

The Office further alleges that the T cells of the '221 patent inherently comprise the endogenous receptor which reacts to an allogeneic cell. As noted above with respect to the '755 patent, there is no evidence of record that a diverse population of T cells have an

endogenous T cell receptor reactive to an allogeneic cell. Accordingly, inherent anticipation of the claimed invention has not been demonstrated in the instant case. That is, even assuming for the sake of argument, that the '221 and '046 patents may disclose T cells having a receptor that reacts to a tumor antigen, neither disclose T cells having a second endogenous receptor that reacts to a cell that is allogeneic to the T cell. Thus, neither the '221 patent, nor the '046 patent, disclose every limitation of the claims.

In view of the foregoing, Applicants respectfully request that the rejection under § 102 in view of the '221 and '046 patents be withdrawn.

Conclusion

The application is considered in good and proper form for allowance, and the Examiner is respectfully requested to pass this application to issue. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned attorney.

Respectfully submitted,

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Redirecting Mouse CTL Against Colon Carcinoma: Superior Signaling Efficacy of Single-Chain Variable Domain Chimeras Containing TCR-ζ vs FcϵRI-γ[1]

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The structurally related TCR-ζ and Fc receptor for IgE (FcϵRI)-γ are critical signaling components of the TCR and FcϵRI, respectively. Although chimeric Ab receptors containing ζ and γ signaling chains have been used to redirect CTL to tumors, a direct comparison of their relative efficacy has not previously been undertaken. Here, in naive T lymphocytes, we compare the signaling capacities of the ζ and γ subunits within single-chain variable domain (scFv) chimeric receptors recognizing the carcinoembryonic Ag (CEA). Using a very efficient retroviral gene delivery system, high and equivalent levels of scFv-ζ and scFv-γ receptors were expressed in T cells. Despite similar levels of expression and Ag-specific binding to colon carcinoma target cells, ligation of scFv-anti-CEA-ζ chimeric receptors on T cells resulted in greater cytokine production and direct cytotoxicity than activation via scFv-anti-CEA-γ receptors. T cells expressing scFv-ζ chimeric receptors had a greater capacity to control the growth of human colon carcinoma in *scid/scid* mice or mouse colon adenocarcinoma in syngeneic C57BL/6 mice. Overall, these data are the first to directly compare and definitively demonstrate the enhanced potency of T cells activated via the ζ signaling pathway. *The Journal of Immunology,* **2001, 166: 182–187.**

Engagement of TCRs and Ig-binding FcR on T lymphocytes and myeloid/NK cells, respectively, are critical events in initiating host immune defense against virus-infected and malignant cells (1, 2). The signaling events downstream of both the TCR and FcR involves rapid phosphorylation of conserved 18-aa Ig tyrosine activation motifs (ITAM),[4] situated in the cytoplasmic domains of these immune receptor complexes and the subsequent activation of Src family and ZAP-70 and Syk kinases (2–8). FcR-γ- and TCR-ζ-chains contain one and three ITAMs, respectively, within their cytoplasmic domains; each ITAM sequence containing a pair of YXXL motifs separated by seven amino acids. It is these variable flanking amino acids that ultimately dictate, through recruitment of specific protein tyrosine kinases, the signaling capability and function of the γ- and ζ-chains (4, 9). The subsequent induction of multiple downstream signaling cascades leads to cell activation and stimulation of critical biological effector functions such as cytolysis, cytokine release, and phagocytosis (8, 10, 11).

Chimeric receptors comprising the γ and ζ cytoplasmic signaling chains fused to an extracellular ligand-binding domain of a heterologous receptor or single-chain Ab have served as effective tools for elucidating and comparing the structure-function relationship of these TCR-ζ and Fc receptor for IgE (FcϵRI)-γ subunits (11–13). In addition, these chimeras have offered excellent new possibilities for designing novel cellular immunotherapies (14–21). We and others have previously shown that upon Ag ligation of these chimeric receptors, expressed in T cell lines (14, 15, 16), NK cells (17, 18), neutrophils (19), and primary T lymphocytes (20, 21), both the γ and ζ subunits can effectively couple to signal transduction pathways. These signaling moieties are thus capable of mediating immune effector functions, equivalent to but independent of the endogenous FcR and TCR, respectively. Comparative studies of the signaling efficacy of γ and ζ in T cell lines, the rat basophilic leukemic cell line, NK cells, and neutrophils have shown the ζ subunit to be more effective than γ in mediating cytolysis and cytokine release in vitro (19, 22). However, to date, comparison of these structurally related signaling subunits in primary T lymphocytes has not been demonstrated, particularly in tumor models in vivo.

We have previously described a highly effective retroviral gene delivery system for efficiently expressing chimeric receptor gene constructs in mouse T lymphocytes (20). These studies demonstrated encouraging efficacy of a single-chain variable domain (scFv)-anti-carcinoembryonic Ag (CEA)-γ chimera in redirecting T cell-mediated rejection of colon carcinoma (20). In this study, we have compared the efficacy of scFv-γ and -ζ chimeric receptors, recognizing the CEA, to redirect T lymphocyte-mediated effector function both in vitro and in vivo. In keeping with our previous findings, T lymphocytes expressing the scFv-γ chimeric receptor were capable of efficiently mediating rejection of colon carcinoma in an Ag-specific manner (20). Importantly however, T lymphocytes expressing the scFv-ζ chimeric receptors consistently demonstrated a greater capacity to mediate cytokine production, direct cytotoxicity, and tumor rejection in vivo. Thus, the TCR-ζ

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[4] Abbreviations used in this paper: ITAM, Ig tyrosine activation motifs; CEA, carcinoembryonic Ag; scFv, single-chain variable domain; B6, C57BL/6.

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molecule was a superior signaling moiety in chimeric receptors redirecting T cell effector function.

Materials and Methods

Cell culture

The human colorectal carcinoma cell lines COLO 205 and Lovo, mouse (C57BL/6 (B6)) colon adenocarcinoma MC-38 and its CEA^+ transfectant, MC-38-CEA2 (Ref. 23; kindly provided by Dr. Jeff Schlom, National Institutes of Health, Bethesda, MD), and the B6 sarcoma cell line 24JK (kindly provided by Dr. Patrick Hwu, National Institutes of Health) were maintained in RPMI 1640 or DMEM at 37°C and 5% CO_2 supplemented with the following additives: 10% (v/v) FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Grand Island, NY). The retroviral packaging cell lines, GP+E86 and PA317 and the fibroblast cell line NIH3T3 were cultured in DMEM with additives. PA317 cells transduced with recombinant retroviral DNA were maintained in DMEM supplemented with 0.5 mg/ml G418 (Life Technologies). Transduced T cells were cultured in DMEM containing 100 U/ml of human rIL-2 (kindly provided by Chiron, Emeryville, CA).

Mice

Inbred B6, BALB/c, and BALB/c *scid/scid* (*scid*) mice were purchased from The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia. Mice of 4–8 wk of age were used in all experiments that were performed according to animal experimental ethics committee guidelines.

Chimeric receptor gene construction

A 767-bp fragment of DNA coding for scFv of anti-CEA and a marker epitope from c-*myc* was amplified by PCR from the vector MFE-23 (24) and subcloned into *XbaI/BstEII*-digested pRSVscFvγR (a kind gift from Zelig Eshhar, Weizmann Institute, Rehovot, Israel). The chimeric gene constructs were composed of the scFv of the anti-CEA mAb, a membrane-proximal hinge region of human CD8 and the transmembrane and cytoplasmic regions of the human FcεRI-γ or TCR-ζ-chains. The scFv anti-CEA chimeric receptors were digested with *Sna*B1/*Xho*I and were subcloned into the *HpaI/Xho*I restriction sites of the retroviral vector, pLXSN (a kind gift from Dusty Miller, Fred Hutchinson Cancer Research Center, Seattle, WA) containing the long terminal repeat and a neomycin resistance gene under the control of an SV40 promoter.

Retroviral gene transfer of mouse spleen T lymphocytes

Stable GP+E86 ecotrophic packaging cell lines expressing either scFv-anti-CEA-γ or -ζ receptors were isolated as described previously (20). GP+E86 clones producing ~10^7 cfu/ml were used for transduction of mouse spleen T lymphocytes. Spleen cells from mice were initially depleted of RBC by hypotonic lysis with NH_4Cl and enriched by passing through a nylon wool syringe as described previously (25). Enriched T lymphocytes (10^7) were then cocultivated for 72 h with 5 × 10^5 viral-producing packaging cells in DMEM supplemented with 4 μg/ml polybrene, 5 μg/ml PHA (Sigma, St. Louis, MO), and 100 U/ml rIL-2. Following cocultivation, T cells were separated from adherent packaging cells, washed with DMEM and cultured in DMEM supplemented with 100 U/ml rIL-2. T cells were subsequently analyzed for transduction efficiency by flow cytometry and used for in vitro assays and in vivo experiments.

Flow cytometry

Detection of cell surface chimeric receptor expression on mouse T lymphocytes was achieved by indirect immunofluorescence with a c-*myc* tag Ab purified from supernatants of mouse 9E10 cells (26), followed by staining with a PE-labeled anti-mouse Ig mAb (Becton Dickinson, San Jose, CA). Cell surface phenotyping of transduced cells was determined by direct staining with Quantum Red-labeled anti-CD4 (clone H129-19; Sigma) and anti-CD8 (clone 53-6.7; Sigma) and PE-labeled anti-TCRβ (clone H57-597; PharMingen, San Diego, CA) mAbs as previously described (20).

Ag-specific binding, cytotoxicity, and cytokine secretion

The binding capacity of gene-modified mouse T lymphocytes was determined in a rosetting assay as described (16). The cytolytic capacity of transduced T cells was determined in a 6-h ^{51}Cr-release assay as described (15). Mouse IFN-γ secretion by scFv-modified mouse T lymphocytes after CEA ligation was detected by ELISA. Transduced T cells (10^6) (transduced with LXSN alone or LXSN plus scFv-anti-CEA-γ or -ζ) were cultured with 10^6 Lovo (CEA^+) or 24JK (CEA^-) cells in 24-well plates for 20 h. Following incubation, supernatants were harvested and spun to remove cell debris. Levels of cytokine production were measured by ELISA (PharMingen) according to the suppliers specifications.

Adoptive transfer models

Three different adoptive transfer tumor models were employed. In the first model, 10^6 mouse 24JK sarcoma cells and/or 5 × 10^6 human COLO 205 colon carcinoma cells were injected s.c. into opposite flanks of groups of 5–10 *scid* mice. Spleen T lymphocytes (5 × 10^6) from BALB/c mice (transduced with LXSN vector alone or LXSN plus scFv-anti-CEA-γ or -ζ) were injected i.v. into groups of 5–10 *scid* mice 6 h (day 0) and 24 h (day 1) after tumor inoculation. In the second model, 10^7 mouse MC-38-CEA2 colon adenocarcinoma cells were injected i.p. into groups of 5–10 B6 mice. Spleen T lymphocytes (5 × 10^4-10^5) from B6 mice (transduced with LXSN vector alone or LXSN plus scFv-anti-CEA-γ or -ζ) were injected i.p. into groups of 5–10 syngeneic mice 6 h (day 0) and 24 h (day 1) after tumor inoculation. In the third model, 5 × 10^6 mouse MC-38 colon adenocarcinoma cells and/or the CEA^+ transfectants, 5 × 10^6 MC-38-CEA2, were injected s.c. into opposite flanks of groups of 5–10 B6 mice. Spleen T lymphocytes (5 × 10^6) from B6 mice (transduced with LXSN vector alone or LXSN plus scFv-anti-CEA-γ or -ζ) were injected i.v. into groups of 5–10 syngeneic mice 6 h (day 0) and 24 h (day 1), or day 3 after tumor inoculation. In the s.c. models, subsequent tumor growth was monitored daily and measured by a caliper square along the perpendicular axes of the tumors. The data were recorded as the mean tumor size (product of the two perpendicular diameters) ± SEM. In the i.p. model, mice were monitored daily for tumor ascites development, indicated by swelling of the abdomen and were culled when obvious signs of tumor growth were noted.

Results

Expression of chimeric scFv-anti-CEA-γ and -ζ receptors in mouse T lymphocytes

The chimeric receptor gene constructs were composed of the scFv (V_H and V_L) regions of the anti-CEA mAb fused to the transmembrane and cytoplasmic regions of the human TCR-ζ or FcεRI-γ signaling chains, via a CD8 hinge (Fig. 1*A*). The scFv-γ and -ζ chimeric gene constructs were subcloned into the retroviral vector pLXSN and high titer-virus-producing GP+E86 clones were used to transduce enriched naive T lymphocytes from BALB/c or B6 mouse spleens as previously described (20). Surface expression of the scFv-anti-CEA receptors in transduced T cells was determined by flow cytometry using an anti-c-*myc* tag mAb directed at the tag epitope located within the extracellular domain of the receptors (Fig. 1, *B–D*). An equivalently high level of expression of the scFv-anti-CEA-γ and -ζ chimeric receptors (ranging between 50 and 80%) was detected on T cells (mean channel fluorescence: scFv-γ = 36.6 ± 5.8, scFv-ζ = 32.8 ± 5.4; n = 8; Fig. 1, *B* and *C*). Expression of either scFv chimeric receptor could not be detected on T cells transduced with the LXSN retroviral vector alone (mean channel fluorescence = 8.80 ± 2.7; n = 8; Fig. 1*D*). Consistent with previous observations (20), preferential proliferation of $CD8^+$ spleen T cells in response to PHA/IL-2 was observed; the transduced T cell populations were consistently >80% $TCR\beta^+CD8^+$.

Effective Ag-specific binding by T cells expressing the scFv-anti-CEA-γ and -ζ chimeric receptors

The ability of mouse T cells expressing either the scFv-anti-CEA-γ or -ζ receptors to specifically bind CEA-expressing target cells was demonstrated in rosetting assays. Transduced BALB/c T cells (scFv-anti-CEA-γ and -ζ) conjugated equally well with the CEA^+ Lovo target cells (40 ± 3% vs 46 ± 2%; n = 4) but not with CEA^- 24JK target cells (data not shown). T cells transduced with pLXSN control vector did not bind either target cell line (data not shown). These data suggested that the level of surface expression and scFv-mediated Ag binding of γ- and ζ-containing chimeric receptors were equivalent.

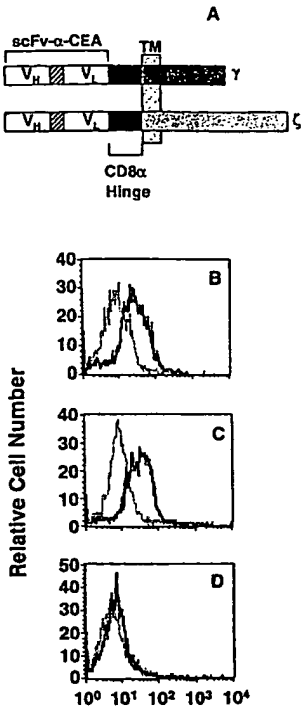

FIGURE 1. Expression of the chimeric scFv-anti-CEA-γ and -ζ receptors in mouse T lymphocytes. *A*, Schematic representation of the scFv-anti-CEA-γ and -ζ receptors. Each construct was composed of the V_H and V_L regions of the anti-CEA mAb joined by a flexible linker, a membrane-proximal hinge region of human CD8 and the transmembrane (TM) and cytoplasmic regions of the human FcεRI-γ- and TCR-ζ-chains. Enriched splenic T cells from BALB/c or B6 mice were transduced by coculture with GP+E86 clones producing the recombinant retroviral vector encoding the anti-CEA-ζ (*B*), the anti-CEA-γ (*C*) gene constructs, or the retroviral vector pLXSN alone (*D*). Cells were stained with the anti-tag mAb and PE-labeled sheep anti-mouse Ig (solid line) or with the PE-labeled secondary alone (dashed line), and receptor expression was analyzed by flow cytometry.

Superior cytokine release and cytotoxicity mediated by scFv-ζ-transduced T cells

The capacity of the scFv-γ- or -ζ-transduced T cells to secrete IFN-γ was compared following specific interaction with CEA^+ target cell lines (Table I). T cells transduced with the scFv-ζ chimera secreted >1.5-fold more IFN-γ than T cells expressing the scFv-γ receptor, following ligation of the CEA^+ Lovo carcinoma cell line (Table I). T lymphocytes transduced with LXSN vector alone secreted <20 pg/ml IFN-γ after interaction with Lovo or 24JK cells. Depletion of $CD8^+$ T cells before interaction with Lovo target cells abolished IFN-γ secretion (data not shown). Human Jurkat T leukemia cells expressing either the scFv-anti-CEA-γ or -ζ receptors also secreted IL-2 in an Ag-specific manner. In particular, after ligation with CEA, Jurkat T cells transduced with scFv-ζ secreted 2-fold more IL-2 than Jurkat transduced with the scFv-γ receptor (data not shown). This data demonstrated the ζ-containing chimera to also be more effective for cytokine production than the scFv-γ chimera in a human T cell line.

Table I. *Optimal IFN-γ secretion by scFv-ζ-expressing mouse T cells after CEA ligation*[a]

Transduced T Cells	Target Cell Line: 24JK (CEA^-) (pg/ml)	Target Cell Line: Lovo (CEA^+) (pg/ml)
LXSN	<20	<20
scFv-ζ	<20	257 ± 23
scFv-γ	<20	132 ± 10

[a] Results are from a representative experiment. Mouse T cells transduced with pLXSN alone or pLXSN encoding the scFv-anti-CEA-γ or -ζ receptors were cultured with 5×10^5 Lovo (CEA^+) or 24JK (CEA^-) for 24 h in 24-well plates at a 1:1 E:T ratio. Harvested supernatants were evaluated for IFN-γ production by ELISA. Results are expressed as pg/ml of IFN-γ production ± SE for duplicate samples.

The capacity of transduced T cells, expressing the scFv-γ or -ζ chimeric receptors to mediate specific target cell lysis was evaluated in standard 6-h ^{51}Cr release assays. BALB/c T cells expressing either the scFv-γ or -ζ receptors were capable of lysing the CEA^+ colon carcinoma cell line COLO 205, but not the CEA^- 24JK sarcoma cell line. Interestingly, BALB/c T cells expressing the scFv-anti-CEA-ζ chimeric receptors were significantly more cytolytic than T cells transduced with the scFv-γ chimera at all E:T ratios (Fig. 2, *A* and *B*). In B6 T lymphocytes (expressing equivalent levels of the scFv-γ or -ζ receptors as detected on transduced BALB/c T cells (data not shown)), the ζ-containing chimera again was shown to have a greater cytolytic capacity than the γ-containing chimera against the CEA^+ MC-38-CEA2 mouse colon adenocarcinoma (Fig. 2*C*). No lysis of the CEA^- parental MC-38 cells was evident in the presence of either effector population (Fig. 2*D*). The antigenic specificity of these responses was further demonstrated by the abrogation of T cell lysis by the addition of an anti-CEA-mAb, but not by an IgG isotype control mAb (data not shown). T cells transduced with the LXSN vector alone were unable to lyse the CEA-expressing and control target cell lines (Fig. 2, *A–D*).

Superior efficacy of scFv-anti-CEA-ζ-transduced T cells in rejecting human colon carcinoma in scid *mice*

The signaling efficacy of the scFv-anti-CEA-γ and -ζ chimeric receptors in stimulating T cell effector function was further evaluated in a human tumor rejection assay in vivo. Both types of

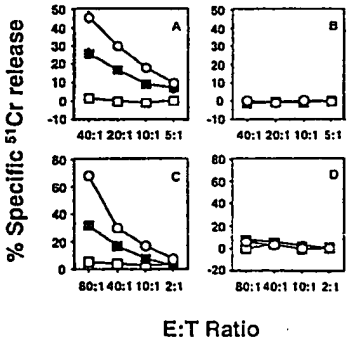

FIGURE 2. Superior efficacy of scFv-anti-CEA-ζ-transduced T cells in mediating Ag-specific lysis of CEA^+ tumor cells by transduced T cells. The cytolytic function of the transduced T cells expressing the scFv-anti-CEA-γ and -ζ receptors was evaluated in a 6-h ^{51}Cr release assay. T cells transduced with the scFv-γ (■) or scFv-ζ (○) chimeric receptors lysed the CEA^+ COLO 205 colon carcinoma cell line (*A*) but not the CEA^- 24JK sarcoma cell line (*B*). T cells transduced with the pLXSN retrovirus alone (□) were unable to lyse the COLO 205 and 24JK cell lines. The lytic activity of enriched splenic T cells from B6 mice transduced with the scFv anti-CEA-γ chimera (■), the scFv anti-CEA-ζ chimera (○), or pLXSN alone (□) was evaluated against the CEA^+ colon adenocarcinoma cell line MC-38-CEA2 (*C*) or CEA^- MC-38 parental cell line (*D*). The spontaneous lysis was <10% in all assays. Results are expressed as specific ^{51}Cr release ± SE (%) for triplicate samples and are representative of at least two experiments.

transduced T cells (5×10^6) were adoptively transferred i.v. into *scid* mice, 6 h (day 0) and 24 h (day 1) after the subcutaneous inoculation of CEA^+ COLO 205 tumor in the right flank and CEA^- 24JK tumor in the left flank. As previously reported (20), the scFv-γ chimeric receptor transduced effector T cells injected i.v. mediated an Ag-specific anti-tumor response against the CEA^+ COLO 205 tumor xenografts, with 2 of 10 tumors completely eradicated (Fig. 3*A*). However, more striking was the effect of the scFv-ζ-transduced T cells, with the complete eradication of 6 of 10 COLO 205 tumors (Fig. 3*A*). Of the mice with tumors not eradicated by treatment with either population of chimera-transduced T cells, only those receiving scFv-ζ-transduced cells displayed a reduced tumor growth rate compared with mice receiving pLXSN-transduced T cells or no treatment. The antigenic-specificity of both scFv chimeric receptors was demonstrated by the complete lack of effect of these transduced T cells on CEA^- 24JK tumors growing in the opposite flank of these mice (Fig. 3*B*). Mice with tumors eradicated by treatment were monitored for between 50 and 100 days after tumor inoculation and all remained tumor free.

Superior efficacy of scFv-anti-CEA-ζ-transduced T cells in controlling syngeneic mouse adenocarcinoma

The efficacy of the scFv-anti-CEA-γ- and -ζ-transduced T lymphocytes was evaluated following adoptive transfer into B6 mice inoculated either i.p. or s.c. with MC-38-CEA2 adenocarcinoma cells. In the first model, i.p. transfer of 5×10^5 T cells transduced with the scFv-anti-CEA-γ receptor, 6 h (day 0) and 24 h (day 1) after i.p. inoculation of MC-38-CEA2 tumor, resulted in 40% survival (4 of 10 mice; Fig. 4). By comparison, intraperitoneal injection of T cells (5×10^5; days 0 and 1) transduced with the scFv-anti-CEA-ζ receptor eradicated tumors, with 70% survival (7 of 10 mice). The superior efficacy of the scFv-ζ chimera was further demonstrated by the 30% survival (3 of 10 mice) of mice i.p. injected with 5×10^4 (days 0 and 1) scFv-ζ-transduced T cells (Fig. 4). The i.p. injection of 5×10^4 (days 0 and 1) scFv-γ-expressing T cells had little anti-tumor effect with no eradication of tumors. Both scFv-anti-CEA-γ- and -ζ-transduced T cells had no effect on the i.p. growth of the MC-38 parental line (data not shown).

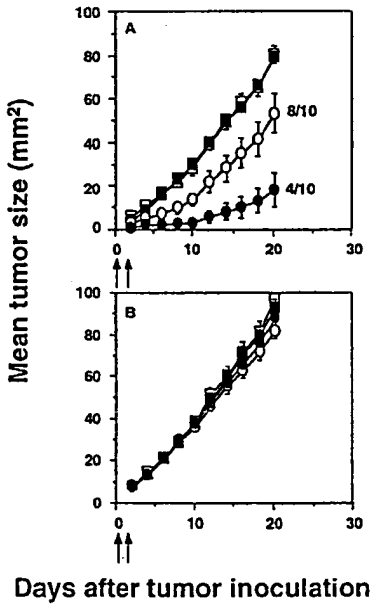

FIGURE 3. Optimal rejection of colon carcinoma in *scid* mice by redirected T cells expressing the scFv-ζ chimera. *A*, The growth of the CEA^+ human COLO 205 colon carcinoma cells in groups of 5–10 *scid* mice injected s.c. into the right flank with 5×10^6 tumor cells. *B*, Mice were additionally injected in the left flank with 10^6 CEA^- 24JK sarcoma cells (■). Mice were injected i.v. with 5×10^6 BALB/c T cells transduced with the pLXSN vector alone (□), scFv-anti-CEA-γ chimera (○), or scFv-anti-CEA-ζ chimera (●) on days 0 and 1 after tumor inoculation. Untreated mice are depicted by filled squares (■) for either COLO 205 (*A*) or 24JK (*B*). For all experiments, results are represented as the mean tumor size (mm^2) ± SE. Arrows depict the days of T cell transfer and the number of tumors not eradicated is shown.

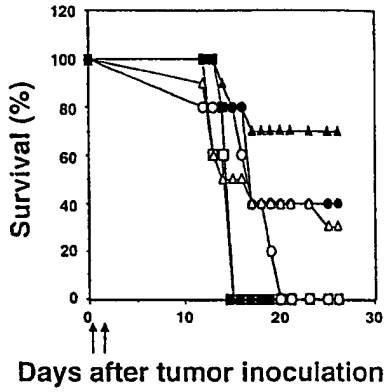

FIGURE 4. Superior anti-tumor response by scFv-ζ-transduced T cells against i.p. syngeneic colon adenocarcinoma. The survival of B6 mice inoculated i.p. with 10^7 MC-38-CEA2 tumor in groups of 5–10 B6 mice. Mice were injected i.p. with 10^6 B6 T cells transduced with the pLXSN vector alone (□), 5×10^4 (○) or 5×10^5 (●) scFv anti-CEA-γ chimera, or 5×10^4 (△) or 5×10^5 (▲) scFv anti-CEA-ζ chimera on days 0 and 1 after tumor inoculation. Untreated mice are depicted by filled squares (■). Results are represented as the percentage of survival and arrows depict the days of T cell transfer.

In the second and more stringent model, the i.v. transfer of T cells transduced with scFv-anti-CEA-γ effectively rejected s.c. MC-38-CEA2 tumor growth in an Ag-specific manner, consistent with our previous findings (20) (Fig. 5, *A* and *B*). Intravenous transfer of 5×10^6 transduced B6 scFv-γ T cells 6 h (day 0) and 24 h (day 1) after tumor inoculation resulted in the complete rejection of MC-38-CEA2 tumor in 3 of 10 mice. However, T cells transduced with scFv-anti-CEA-ζ receptor were more effective, with 6 of 10 mice free of MC-38-CEA2 tumor (Fig. 5*A*). Again, the scFv-ζ-transduced T cells were also more efficient at inhibiting the growth of tumors escaping rejection. The antigenic-specificity of transduced T cells was maintained in this model with no effect on the growth of the CEA^- parental MC-38 tumor in the opposite flank of these mice (Fig. 5*B*).

While we have demonstrated effective early treatment of human colon carcinoma and mouse adenocarcinoma using scFv-anti-CEA-γ-transduced T cells, these T cells were previously only found to be moderately effective against 3-day established tumors (20). We thus compared the efficacy of scFv-anti-CEA-γ- or -ζ-transduced T cells against established MC-38-CEA2 tumors (Fig. 6*A*). Although no complete tumor eradications were achieved, the scFv-anti-CEA-ζ-transduced T cells compared favorably, with a clear improvement in the control of tumor growth (up to a 2-fold reduction in mean tumor size 5–14 days after tumor inoculation) above that observed with scFv-anti-CEA-γ-transduced T cells. Growth of the CEA^- parental MC-38 tumor in the opposite flank of these mice was not affected (Fig. 6*B*).

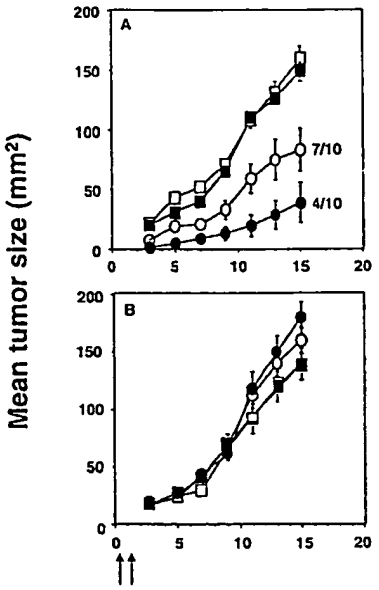

FIGURE 5. Transduced T cells expressing the scFv-anti-CEA-ζ chimera are more efficient at mediating eradication of s.c. syngeneic colon adenocarcinoma. The s.c. growth of 5×10^6 (CEA^+) MC-38-CEA2 (right flank) (*A*) and 10^6 (CEA^-) MC-38 (left flank) (*B*) colon adenocarcinoma cell lines in groups of 5–10 B6 mice. Mice were injected i.v. with 5×10^6 B6 T cells transduced with the pLXSN vector alone (■), scFv anti-CEA-γ chimera (○), or scFv anti-CEA-ζ chimera (●) on days 0 and 1 after tumor inoculation. Untreated mice are depicted by filled squares (■) for either MC-38-CEA2 (*A*) or MC-38 (*B*).

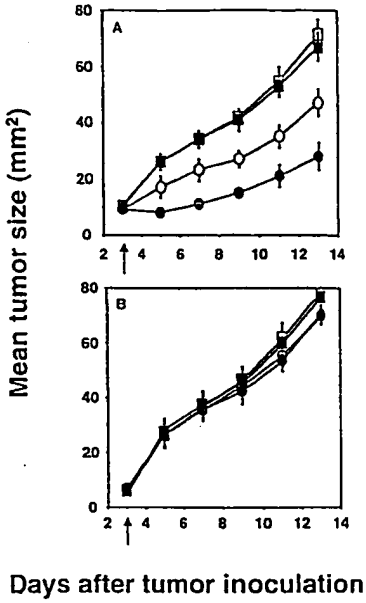

FIGURE 6. Optimal growth inhibition of established s.c. syngeneic colon adenocarcinoma by scFv-anti-CEA-ζ-transduced T cells. The s.c. growth of 5×10^6 (CEA^+) MC-38-CEA2 (right flank) (*A*) and 10^6 (CEA^-) MC-38 (left flank) (*B*) colon adenocarcinoma cell lines in groups of 5–10 B6 mice. Mice were injected i.v. with 5×10^6 B6 T cells transduced with the pLXSN vector alone (□), scFv-anti-CEA-γ chimera (○), or scFv-anti-CEA-ζ chimera (●) on day 3 after tumor inoculation. Untreated mice are depicted by filled squares (■) for either MC-38-CEA2 (*A*) or MC-38 (*B*).

Discussion

Despite existing as subunits of multicomponent surface receptors, the TCR-ζ and FcεRI-γ signaling chains have the capacity to mediate critical biological effector cell activities, equivalent to but independent of the TCR and FcγR, respectively (12, 27). Most useful has been their incorporation into chimeric immune receptors that provide effective tools for designing new cellular immunotherapies for cancer. By linking various signal transduction moieties to a constant extracellular binding domain that specifically regulates effector cell recognition of tumors, we have been able to assess the relative activity of each cytoplasmic domain in stimulating T cell function. Although both the FcεRI-γ- and TCR-ζ-chains have previously been shown to be capable of autonomous activation of T cells (14–21), the signaling efficacy of the γ and ζ subunits has never been directly compared in vivo using primary T cell effectors. Importantly, in this study, high and equivalent expression of both the scFv-ζ and scFv-γ chimeric receptors was achieved in mouse T lymphocytes using retroviral gene transduction, and we have clearly demonstrated the TCR-ζ molecule to be a more effective activator of T cell anti-tumor function than FcεRI-γ. In particular, the significant improvement in the anti-tumor activity of scFv-ζ-transduced T cells against established tumor grafts was encouraging and efforts to further improve chimera design can be adequately tested in these tumor models. Although these tumor models suggest scFv-transduced T cells can prevent tumor initiation at a distant site, the eradication of more established tumors and their metastases is the ultimate goal of this approach.

Although primarily associated with the FcγRI in neutrophils and macrophages (28, 29), expression of FcεRI-γ has been detected in the cytotoxic T cell line, CTLL (30), and in T cell populations such as early thymocytes (31), $NK1.1^+$ thymocytes and T cells (32, 33), and lymphokine-activated $\gamma\delta$-TCR^+ T cells (34). These studies support a potentially broad biological role for γ in T cell-mediating effector activity and thus provided scope for comparing the signaling efficacy of the γ and ζ signaling moieties in primary T lymphocytes. The observation that the TCR-ζ-chain was a superior signal transducer in mouse T lymphocytes was also consistent with previous in vitro studies performed in T cell lines, neutrophils, and NK cell effector populations (19, 22). The observation that ζ was more effective than γ may not be surprising given that the multimerization of ITAMs in the ζ-chain may provide a potential means of signal amplification, which may increase the sensitivity of the scFv-ζ chimera to ligand stimulation (35–37). A previous study has shown the qualitative differences within γ and ζ to account for the markedly more efficient phagocytic signaling capability of the γ subunit (9); however, T cell-mediated phagocytosis was not assessed in this study. An interesting future experiment will be to construct a scFv-ζ chimera composed of a single ITAM to determine whether ITAM multimerization normally provides the scFv-ζ chimera superior signaling efficacy compared with the single ITAM containing scFv-γ chimera. Previously, we demonstrated the critical importance of both direct cytotoxicity mediated by perforin and T cell IFN-γ production to effective tumor control in vivo (20). Importantly, in this study it was shown that IFN-γ was not required for the cytotoxic capacity of scFv-transduced T cells, since those from IFN-γ-deficient mice were as lytic as effectors from wild-type mice. In this light, the greater direct cytolysis and IFN-γ secretion triggered by the ζ-chain chimera in T cells may at least in part explain the enhanced in vivo activity of these transduced T cells. It remains to be assessed whether ζ-containing chimeras provide a greater proliferative and more effective survival signal than scFv-γ receptors. Mice cured of tumor did not resist subsequent tumor rechallenge (data not shown), suggesting that long-lived effector cells and memory cells were not present.

However, repeated adoptive transfer, coadministration of T cell help/cytokines and/or modified scFv chimera design will be pursued to strive for more effective and sustained tumor protection.

Given the design of the γ- and ζ-containing chimeras used in this study, the ability to harness the signaling machinery of the TCR may hypothetically account for the superior signaling capabilities of the ζ subunit in the mouse T lymphocytes. Each chimeric receptor contained the complete γ- or ζ-chain transmembrane sequence, sufficient to enable the dimerization of the chimeras with endogenous γ- or ζ-chains of the TCR expressed on the transduced T cells (30, 38). In our tumor model, antigenic specificity of the redirected CTL was demonstrated by the rejection of CEA^+ colon carcinomas but not CEA^- tumor inoculated in the opposite flank of these mice. However, we cannot discount the potential involvement of endogenous TCR signaling activity in these antitumor responses, initiated by Ag ligation of the chimeric scFv receptors. Importantly, ligation of CEA did not appear to cotrigger any endogenous TCR reactivity with self tissues in the syngeneic mouse tumor model. The higher binding affinity of scFv-receptors, compared with native TCR, for ligand could potentially influence the potency of the biochemical responses mediated by particular T cell effector populations (39, 40). We are currently assessing scFv chimera expression in TCR-transgenic T cells to determine whether endogenous T cell activity is triggered or dampened. Neither syngeneic nor xenogeneic CEA^+ tumor models assessed autoimmunity via CEA ligation, since this Ag is tumor specific in B6 or *scid* mice, respectively. However, autoimmunity is a potential problem for this scFv approach in general, while tumor-restricted Ags remain limiting. In the future, models should be established that take this reality into consideration although bystander autoimmunity is only most accurately defined in clinical trials. Unlike vaccination strategies, if autoimmunity did arise, adoptively transferred scFv-expressing T cells could be tailored with suicide genes.

With the rapid development of new lentiviral vector systems, the transfer of scFv chimeras into nondividing and/or human T lymphocytes is now a reality. Designed chimeric immune receptors, containing cytoplasmic domains that can optimally stimulate T cell effector function, proliferation, and survival, can be tested in these settings and ultimately be exploited to enhance the potency and safety of this adoptive immunotherapy in the clinic.

References

1. Klausner, R. D., and L. E. Samelson. 1991. T cell antigen activation pathways: the tyrosine kinase connection. *Cell 64:875.*
2. Weiss, A., and D. R. Littman. 1994. Signal transduction by lymphocyte antigen receptors. *Cell 76:263.*
3. Zhang, W., J. Sloan-Lancaster, J. Kitchen, R. P. Tribble, and L. E. Samelson. 1998. LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. *Cell 92:83.*
4. Zoller, K. E., I. A. MacNeil, and J. S. Brugge. 1997. Protein tyrosine kinases Syk and ZAP-70 display distinct requirements for Src family kinases in immune response receptor signal transduction. *J. Immunol. 158:1650.*
5. Cambier, J. C. 1995. Antigen and Fc receptor signaling: the awesome power of the immunoreceptor tyrosine-based activation motif (ITAM). *J. Immunol. 155:3281.*
6. Wange, R. L., and L. E. Samelson. 1996. Complex complexes: signaling at the TCR. *Immunity 5:197.*
7. El-Hillal, O., T. Kurosaki, T. Yamamura, J. P. Kinet, and A. M. Scharenberg. 1997. Syk kinase activation by a src kinase-initiated activation loop phosphorylation chain reaction. *Proc. Natl. Acad. Sci. USA 94:1919.*
8. Kolanus, W., C. Romeo, and B. Seed. 1993. T cell activation by clustered tyrosine kinases. *Cell 74:171.*
9. Park, J. G., and A. D. Schreiber. 1995. Determinants of the phagocytic signal mediated by the type IIIA Fcγ receptor, FcRIIIA: sequence requirements and interaction with protein kinases. *Proc. Natl. Acad. Sci. USA 92:7381.*
10. Indik, Z., J. G. Park, S. Hunter, and A. D. Schreiber. 1995. The molecular dissection of Fcγ receptor mediated phagocytosis. *Blood 86:4389.*
11. Wegener, A. K., and F. Letourneur. 1992. The T cell receptor/CD3 complex is composed of at least two autonomous transduction modules. *Cell 68:83.*
12. Irving, B. A., and A. Weiss. 1991. The cytoplasmic domain of the T cell receptor ζ-chain is sufficient to couple to receptor-associated signal transduction pathways. *Cell 64:891.*
13. Romeo, C., and B. Seed. 1991. Cellular immunity to HIV activated by CD4 fused to T cell or Fc receptor polypeptides. *Cell 64:1037.*
14. Eshhar, Z., T. Waks, G. Gross, and D. G. Schindler. 1993. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the γ and ζ subunits of the immunoglobulin and T-cell receptors. *Proc. Natl. Acad. Sci. USA 90:720.*
15. Darcy, P. K., M. H. Kershaw, J. A. Trapani, and M. J. Smyth. 1998. Expression in cytotoxic T lymphocytes of a single-chain anti-carcinoembryonic antigen antibody: redirected Fas ligand-mediated lysis of colon carcinoma. *Eur. J. Immunol. 28:1663.*
16. Haynes, N. H., M. J. Smyth, M. H. Kershaw, J. A. Trapani, and P. K. Darcy. 1999. Fas-ligand mediated lysis of erbB-2 expressing tumor cells by redirected cytotoxic T lymphocytes. *Cancer Immunol Immunother. 47:278.*
17. Hege, K. M., M. H. Cooke, M. H. Finer, K. M. Zsebo, and M. R. Roberts. 1996. Systemic T cell-independent tumor immunity after transplantation of universal receptor-modified bone marrow into SCID mice. *J. Exp. Med. 184:2261.*
18. Tran, A. C., R. B. Zhang, and M. R. Roberts. 1994. Chimeric-ζ receptors direct human natural killer effector function to permit killing of NK resistant tumor cells and HIV-infected T lymphocytes. *J. Immunol. 155:1000.*
19. Roberts, M. R., K. S. Cooke, A. Tran, K. A. Smith, W. Y. Lin, M. Wang, T. J. Dull, D. Farson, K. M. Zsebo, and M. H. Finer. 1998. Antigen-specific cytolysis by neutrophils and NK cells expressing chimeric immune receptors bearing ζ and γ signaling domains. *J. Immunol.* 161:375.
20. Darcy, P. K., N. M. Haynes, M. B. Snook, J. A. Trapani, L. Cerruti, S. M. Jane, and M. J. Smyth. 2000. Redirected perforin-dependent lysis of colon carcinoma by ex-vivo genetically engineered CTL. *J. Immunol. 164:3705.*
21. Altenschmidt, U., E. Klundt, and B. Groner. 1997. Adoptive transfer of in vitro-targeted, activated T lymphocytes results in total tumor regression. *J. Immunol. 159:5509.*
22. Letourneur, F., and R. D. Klausner. 1991. T-cell and basophil activation through the cytoplasmic tail of T cell receptor ζ family proteins. *Proc. Natl. Acad. Sci. USA 88:8905.*
23. Horan Hand, P., P. F. Robbins, M. L. Salguller, D. J. Poole, and J. Schlom. 1993. Evaluation of human carcinoembryonic-antigen (CEA)-transduced and non-transduced murine tumors as potential targets for anti-CEA therapies. *Cancer Immunol Immunother. 36:65.*
24. Chester, K. A., R. H. Begent, L. Robson, P. Keep, R. B. Pedley, J. A. Bodin, G. Boxer, A. Green, G. Winter, O. Cochet, et al. 1994. Phage libraries for clinically useful antibodies. *Lancet 343:455.*
25. Coligan, J. E., A. M. Kruisbeer, D. H. Margulies, E. M. Shevach, and W. Strober. 1993. T cell enrichment by nonadherence to nylon. In *Current Protocols in Immunology.* Wiley, New York, p. 3.2.1.
26. Evan, G. I., G. K. Lewis, G. Ramsey, and J. M. Bishop. 1985. Isolation of monoclonal Ab specific for human c-*myc* proto-oncogene product. *Mol. Cell Biol. 5:3610.*
27. Hwu, P., G. E. Shater, D. G. Treisman, G. Schindler, G. Gross, R. Consher, S. A. Rosenberg, and Z. Eshhar. 1993. Lysis of ovarian cancer cells by human lymphocyte redirected with a chimeric gene composed of an antibody variable region and the Fc receptor γ-chain. *J. Exp. Med. 178:361.*
28. Scholl, P. R., and R. S. Geha. 1993. Physical association between the high affinity IgG receptor (FcγRI) and the γ subunit of the high affinity IgE receptor (FcεRIγ). *Proc. Natl. Acad. Sci. USA 9:8847.*
29. Kuster, H., H. Thompson, and J. P. Kinet. 1990. Characterization and expression of the gene for the human Fc receptor γ subunit: definition of a new gene family. *J. Biol. Chem. 265:6448.*
30. Orloff, D. G., C. Ra, S. J. Frank, R. D. Klausner, and J. P. Kinet. 1990. Family of disulfide-linked dimers containing the ζ- and η-chains of the T-cell receptor and the γ-chain of Fc receptors. *Nature 347:189.*
31. Heiken, H., R. J. Schulz, E. Ravetch, E. Reinherz, and S. Koyasu. 1996. T lymphocyte development in the absence of FcεRIγ subunit: analysis of thymus-development and independent αβ and γδ pathways. *Eur. J. Immunol. 26:1935.*
32. Curnow, S. J., C. Boyer, M. Buferne, and A. M. Schmitt-Verhulst. 1995. TCR-associated ζ-FcεRIγ heterodimers on CD4-CD8-NK1.1$^+$ T cells selected by specific class I MHC antigen. *Immunity 3:427.*
33. Koyasu, S., L. D'Adamino, A. R. N. Amlanandam, S. Abraham, C. R. Clayton, and E. L. Reinherz. 1992. T cell receptor complexes containing FcεRIγ heterodimers in lieu of CD3ζ and CD3η components: a novel isoform expressed in large granular lymphocytes. *J. Exp. Med. 175:703.*
34. Qian, D., A. I. Sperling, D. W. Lanchi, Y. Tatsumi, T. A. Burrett, J. A. Bluestone, and F. W. Fitch. 1993. The γ-chain of the high-affinity receptor for IgE is a major functional subunit of the T-cell antigen receptor complex in γδ lymphocytes. *Proc. Natl. Acad. Sci. USA 90:11875.*
35. Irving, B. A., A. C. Chan, and A. Weiss. 1993. Functional characterisation of a signal transducing motif present in the T cell receptor ζ-chain. *J. Exp. Med. 177:1093.*
36. Wegener, A. M., F. Letourneur, A. Hoeveler, T. Brocker, F. Luton, and B. Malissen. 1992. The T cell receptor/CD3 complex is composed of at least two autonomous transduction modules. *Cell 68:83.*
37. Geiger, T. L., D. Leitenberg, and R. A. Flavell. 1999. The TCR ζ-chain immunoreceptor tyrosine-based activation motifs are sufficient for the activation and differentiation of primary T lymphocytes. *J. Immunol. 162:5931.*
38. Rutledge, T., P. Cosson, N. Manolios, J. Bonifacino, and R. Klausner. 1992. Transmembrane helical interactions: ζ-chain dimerization and functional association with the T cell receptor. *EMBO J. 11:3245.*
39. Shores, E. W., M. Ono, T. Kawabe, C. L. Sommers, T. Tran, K. Lui, M. C. Udey, J. Ravetch, and P. E. Love. 1998. T cell development in mice lacking all T cell receptor ζ family members (ζ, η, and FcεRIγ). *J. Exp. Med. 187:1093.*
40. Lanzavecchia, A., G. Lezzi, and A. Viola. 1999. From TCR engagement to T cell activation: A kinetic view of T cell behaviour. *Cell. 96:1.*

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Prevention of Mammary Tumors with a Chimeric HER-2 B-cell Epitope Peptide Vaccine[1]

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ABSTRACT

Synthetic peptide vaccines targeting B-cell epitopes of the extracellular domain of the HER-2 oncoprotein were evaluated for their capacity to elicit HER-2-specific antibodies with antiproliferative activity. Several HER-2 B-cell epitopes were identified by computer-aided analysis of protein antigenicity, and selected B-cell epitopes were synthesized colinearly with a promiscuous T-helper epitope (208–302) derived from the measles virus fusion protein at either the NH_2 or COOH terminus linked via a four-residue turn sequence (GPSL). In addition, one epitope sequence, 628–647, was mutated to optimize disulfide pairing to mimic the native HER-2 receptor. All of the four selected epitopes elicited high-titered antibodies in outbred rabbits with exceptionally high titers for MVF-HER-2(628–647). These antibodies were cross-reactive with the native HER-2 receptor. Antibodies elicited by MVF HER-2(628–647) inhibited proliferation of human HER-2-overexpressing breast cancer cells *in vitro* and caused their antibody-dependent cell-mediated cytotoxicity. Furthermore, immunization with MVF-HER-2(628–647) prevented the spontaneous development of HER-2/neu-overexpressing mammary tumors in 83% of transgenic mice. The engineered, chimeric peptide B-cell immunogen MVF-HER-2(628–647) may have applications in the prevention of HER-2-overexpressing cancers.

INTRODUCTION

HER-2 is a M_r 185,000 transmembrane phosphoglycoprotein encoded by the *erb*B-2 gene, the human homologue of the rat proto-oncogene neu. HER-2 is a member of the EGFR[4] (EGFR/*erb*B-1) family. It is composed of an ECD that is cysteine rich and has several glycosylation sites and an intracellular domain with a highly conserved tyrosine kinase (1, 2). Although a direct ligand for HER-2 has not been described, it has been shown to function as a preferential heterodimerization signaling partner with EGFR, HER-3, and HER-4 by providing a low-affinity ligand binding site (3, 4). In humans, HER-2 is expressed in fetal tissues and at low levels in normal tissues of adults (5). Overexpression of HER-2 is associated with 20–30% of breast and ovarian cancers and, to a lesser extent, with adenocarcinoma of uterus, cervix, fallopian tube, and endometrium (6–8). In patients with breast cancer, HER-2 overexpression is an independent predictor of survival; it is associated with poor prognosis, aggressive disease, and resistance to chemotherapy and hormone therapy (8–10). How HER-2 alters the growth of normal or cancer cells is not entirely clear. HER-2 overexpression may provide tumors with a selective growth advantage through increased utilization of stromal-derived epidermal growth factor-like growth factors or ligand-independent receptor homodimerization (11, 12).

HER-2 is an attractive target for immunotherapeutic approaches. Antibodies directed against the ECD of HER-2 have been shown to confer inhibitory effects on tumor growth *in vitro* and in animal models (13–18). In Phase II and Phase III clinical trials, a recombinant humanized anti-HER-2 monoclonal antibody, Trastuzumab, produced an overall response rate of 15% as a single agent in patients with metastatic HER-2-overexpressing breast cancers and has been shown to improve survival when combined with cytotoxic chemotherapeutics (19–21). The molecular mechanisms underlying these growth-inhibitory effects are not well understood. Initial studies showed that antibodies to HER-2 could cause receptor internalization and degradation with reduced phosphorylation resulting in the inhibition of tumor cell growth (14, 22, 23). There is evidence that HER-2 antibodies can block heterodimer formation, interfere with ligand binding, or trigger apoptosis (24–26). HER-2 antibodies also mediate complement-dependent cytotoxicity and/or ADCC (27–29).

Active specific immunotherapy offers the possibility of generating sustained anti-HER-2 immune responses and is potentially more effective than passive approaches, particularly when the application is primary or secondary cancer prevention. A number of vaccine approaches targeting p185 HER-2 or the HER-2 ECD have been evaluated. Strain NFS mice immunized with a vaccinia virus recombinant that expresses the ECD rat *neu* developed a protective antibody response against subsequent challenge with *neu*-transformed NIH 3T3 cells (30). However, immunization of BDIX rats with the same immunogen did not result in antibody response, nor did it inhibit the growth of syngeneic neu-expressing B104 neuroblastoma cells, suggesting that this strategy was insufficient to induce immune responses in the rat. A polysaccharide-oncoprotein complex vaccine consisting of the 147 NH_2-terminal amino acids of HER-2 ECD complexed with cholesteryl group-bearing mannan and pullulan induced cellular and humoral immune responses that mediated rejection of HER-2-expressing sarcomas in BALB/c mice (31). Partial protection was shown in rat *neu* transgenic mice destined to develop mammary tumors by immunizing them with either a purified rat neu ECD (32) or neu-transfected allogeneic mouse fibroblasts (33).

Despite the evidence presented above, it is not entirely clear whether effective immune responses can be generated in humans using cell- or protein-based vaccine strategies targeting p185 HER-2 or the HER-2 ECD because HER-2 is a nonmutated "self" antigen. Moreover, some antibodies elicited to HER-2 have been shown to stimulate rather than inhibit the growth of human tumors, and HER-2 vaccines presenting multiple epitopes could potentially elicit a mixture of counterproductive humoral responses (22). Immunization to self tumor antigens may require a vaccine design that targets a portion of the protein rather than whole protein domains of the antigen. There may be advantages to the use of subunit peptide-based immunogens when targeting HER-2 not only to elicit a desired immune response but also to circumvent tolerance to native protein. Disis *et al.* (34) have shown that immunization of rats with multiple T-helper peptides derived from the rat neu protein elicited strong humoral and CD4+ responses; in contrast, immunization with purified whole rat neu

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[4] The abbreviations used are: EGFR, epidermal growth factor receptor; ECD, extracellular domain; ADCC, antibody-dependent cell-mediated cytotoxicity; MVF, measles virus fusion epitope sequence 208–302; PBMC, peripheral blood mononuclear cell; PBT, PBS containing 0.05% Tween 20 and 1% horse serum.

protein in parallel experiments failed to elicit detectable immune responses. Recently, these investigators also showed that immunization of breast and ovarian cancer patients with multiple HER-2 peptides selected for binding to MHC class II molecules elicited both peptide- and protein-specific T-helper cell responses (35). Whether immune responses elicited by peptide immunogens incorporating human HER-2 T-helper cell epitopes will be of sufficient potency to mediate antitumor activity in humans is not known. The genetic MHC-restricted stimulatory activity of human self-peptides corresponding to T-cell epitopes is also a major obstacle to developing T-cell peptide vaccine approaches for use in an "outbred" human population.

We hypothesized that a rationally designed peptide vaccine targeting specific B-cell determinants from the HER-2 ECD could induce antibodies capable of inhibiting the growth of HER-2-expressing cancers. To augment antibody responses and overcome MHC genetic polymorphism, "promiscuous" T-helper peptide epitopes from a nonhuman molecule may be incorporated. The B-cell HER-2 epitopes were designed with a minimal number of point mutations to facilitate folding of the peptide into a stable conformation to mimic the native protein structure. They were synthesized colinearly with a promiscuous T-helper cell epitope derived from amino acid sequence 288–302 of the measles virus fusion protein. MVF has previously been shown to interact with several distinct human MHC class II alleles (36). Furthermore, we have shown that MVF-conjugated B-cell epitope peptide constructs could be used to bypass certain haplotype-restricted immune responses and provide broad immunogenicity in a large number of individuals typical of an outbred population (37–42). Here we demonstrate that chimeric peptide immunogens targeting a single HER-2 B-cell epitope and incorporating a promiscuous T-helper epitope are capable of eliciting high-titered, native receptor-specific humoral responses in outbred rabbits. Antibodies elicited by one of these immunogens, MVF HER-2(628–647), could selectively inhibit the growth of HER-2-overexpressing cells. Moreover, active immunization with this peptide construct prevented the development of tumors in a transgenic mouse model of HER-2/neu mammary tumorigenesis.

MATERIALS AND METHODS

B-cell Epitope Prediction and Peptide Synthesis. The selection of candidate B-cell epitopes expressed within the human HER-2 ECD was accomplished by computer-aided analysis using various correlates of protein antigenicity as reviewed by Kaumaya *et al.* (43). The basic premise is that algorithms used in this analysis will always locate regions that are surface-exposed on the protein and therefore most likely to be involved in antibody binding. Selected B-cell epitopes were synthesized colinearly with the T-helper epitope MVF using a 4-residue amino acid linker (GPSL) as described previously (44) either on a Milligen/Biosearch 9600 peptide synthesizer (Bedford, MA) or a multiple peptide synthesizer (Model 396; Advance Chemtech, Louisville, KY) using a 4-methylbenzhydrylamine resin as the solid support (substitution, 0.54mm/g). The Fmoc/t-butyl synthetic method was used, using 4-(hydroxymethyl) phenoxyacetic acid as the linker. After the final deprotection step, protecting groups and peptide resin bond were cleaved with 90% triflouroacetic acid, 5% anisole, 3% thioanisole, and 2% ethanedithiol. The crude peptides were purified by reverse-phase high-performance liquid chromatography and were >95% pure before immunization. The identity of the peptides was confirmed by krotos IV MALDI-TOF matrix-assisted laser desorption ionization-time of flight spectrometry at the Complex Carbohydrate Research Center (Athens, GA).

Immunization of Rabbits and Transgenic Mice. Female New Zealand White rabbits were obtained from Mohican Valley Rabbitry (Loudenville, OH). Pairs of rabbits were immunized s.c. at multiple sites with a total of 1 mg of each of the four chimeric peptides (Table 1) emulsified in complete Freund's adjuvant. Subsequent booster injections, 1 mg and 500 μg of the peptide in PBS, were given 3 and 6 weeks after the primary immunization. Sera were collected, and complement was inactivated by heating to 56°C for 30 min. High-titered sera were purified on a protein A/G-agarose column (Pierce, Rockford, IL), and eluted antibodies were concentrated and exchanged in PBS using M_r 100,000 cutoff centrifuge filter units (Millipore, Bedford, MA). The concentration of antibodies was determined by the Coomassie plus protein assay reagent kit (Pierce). Transgenic mice (strain N202) overexpressing the rat *neu* gene under the transcriptional control of the mouse mammary tumor virus promoter were purchased from The Jackson Laboratory (Bar Harbor, ME). Groups of six transgenic mice, each 4–6 weeks old, were immunized separately with 100 μg of HER-2(115–136) MVF, HER-2(410–429) MVF, and MVF HER-2(628–647). The peptides were dissolved in PBS with 100 μg of muramyl dipeptide adjuvant *N*-acetyl-glucosamine-3 yl-acetyl L-alanyl-D-isoglutamine and emulsified (50:50) in Squalene/Arlacel A oil (4:1) as described elsewhere (45). Nine mice were injected with MVF/*N*-acetyl-glucosamine-3 yl-acetyl L-alanyl-D-isoglutamine emulsion as immunized controls. Boosters were given s.c. after 4, 8, 16, and 24 weeks. Two more boosters were also given at 32 and 40 weeks with only MVF HER-2(628–647) to sustain the high-titered immune responses. Mice were retro-orbitally bled monthly for antibody titer determination. Tumor size (length and width) was measured with vernier calipers. Individual tumors volumes were calculated by the formula (length × width^2/2).

ELISA. The 96-well plates were coated with 100 μl of antigen at 2 μg/ml in PBS overnight at 4°C. Nonspecific binding sites were blocked for 1 h with 200 μl of PBS-1% BSA, and plates were washed with PBT. Rabbit antiserum (1:500) or mouse antiserum (1:50) in PBT was added to antigen-coated plates in duplicate wells, serially diluted 1:2 in PBT, and incubated for 2 h at room temperature. After washing the plates, 100 μl of 1:500 goat antirabbit or goat antimouse IgG conjugated to horseradish peroxidase (Pierce) were added to each well and incubated for 1 h. After washing, the bound antibody was detected using 50 μl of 0.15% H_2O_2 in 24 mM citric acid and 5 mM sodium phosphate buffer (pH 5.2) with 0.5 mg/ml 2,2′-aminobis(3-ethylbenzthiazoline-6-sulfonic acid) as the chromophore. Color development was allowed to proceed for 10 min, and the reaction was stopped with 25 μl of 1% SDS. Absorbance was determined at 410 nm using a Dynatech MR700 ELISA reader (Chantilly, VA). Titers were defined as the highest dilution of sera with an absorbance of greater than 0.2 after subtracting the background.

Mouse Isotyping. MVF HER-2(628–647) antibodies raised in transgenic mice were typed using a Mouse Typer Sub-Isotyping Kit (Bio-Rad, Hercules, CA). The assay was performed according to the manufacturer's instructions, except that a 1:1000 dilution of goat antirabbit IgG horseradish peroxidase conjugate was used.

Cell Culture. All cell culture media, FCS, and supplements were purchased from Life Technologies, Inc. (Grand Island, NY). The human breast adenocarcinoma cell lines SK-BR-3 and BT-474 overexpressing HER-2 and the rat *neu*-overexpressing fibroblast cell line DHFR-G8 were purchased from American Type Culture Collection (Manassas, VA) and maintained according to the suppliers' guidelines. CAV-1 was derived from a fresh colon tumor

Table 1 *Amino acid sequences and the structural attributes of chimeric B-cell epitope constructs used in this work*

MVF sequence is italicized. *N*-linked glycosylation sites are shown in bold. The cysteine to glycine mutation in HER-2(628–647) is underlined.

B-cell epitope constructs	Amino acid sequence	Predicted secondary structures	M_r
HER-2(115–136) MVF	H_2N-AVLDNGDPLNNTTPVTGASPGG-GPSL-*KLLSLIKGVIVHRLEGVE*-COOH	117–119 turn; 123–129 β sheet	4407
HER-2(376–395) MVF	H_2N-FLPESFDGDPASNTAPLQPE-GPSL-*KLLSLIKGVIVHRLEGVE*-COOH	376–379 & 389–395 α-helix; 382–384 turn	4472
HER-2(410–429) MVF	H_2N-LYISAWPDSLPDLSVFQNLQ-GPSL-*KLLSLIKGVIVHRLEGVE*-COOH	421–429 β sheet	4646
MVF HER-2(628–647)	H_2N-*KLLSLIKGVIVHRLEGVE-LSPG-*IN**G**THSCVDLDDKGCPAEQR-COOH	632–634 β sheet; 635–637 & 642–646 α helix; 638–639 turn	4498

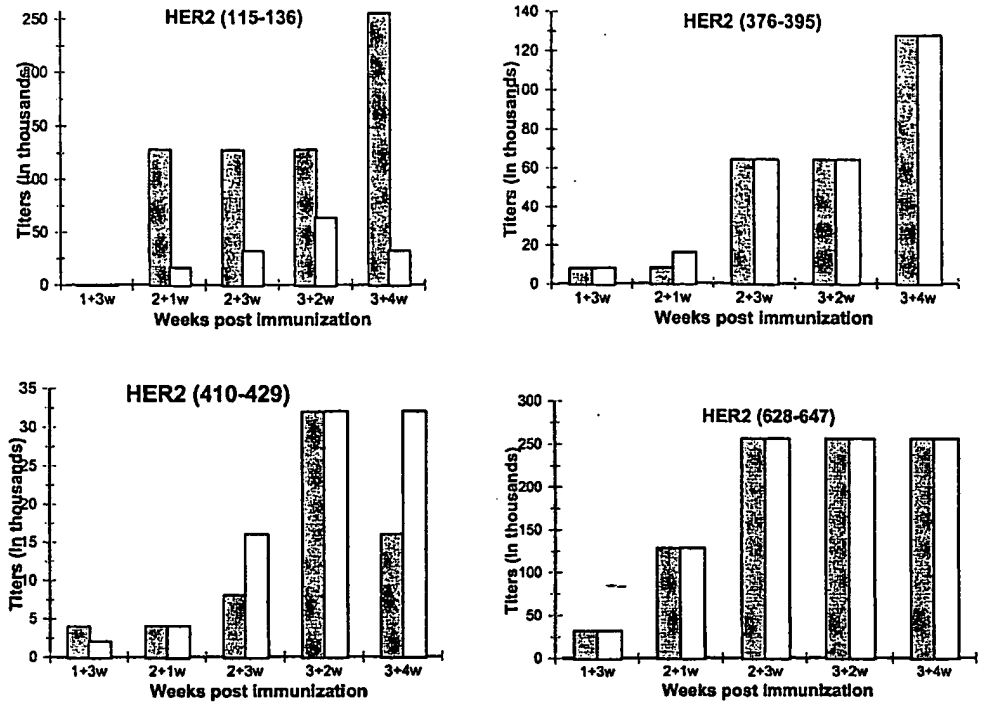

Fig. 1. Immune responses to HER-2 peptide constructs (indicated at the *top* of each panel) in two different rabbits (■ and □) were determined by ELISA. Designation is shown on the *X* axis (*e.g., 1+3w* on the *X* axis indicates sera collected 3 weeks after the first immunization).

specimen that was cryopreserved and subsequently cultured. This cell line does not express detectable levels of HER-2/*neu*. CAV-1 was maintained in RPMI 1640 with 10% FCS and L-glutamine.

Immunoprecipitation and Western Blotting. SK-BR-3 or DHFR-G8 cells (1×10^7) suspended in 100 μl of HBSS per sample were lysed in 1 ml of ice-cold 0.5% NP40 lysis buffer [150 mM NaCl, 50 mM Tris (pH 8), 10 mM EDTA, 10 mM Na PP_i, 10 mM sodium fluoride, 1% NP40, and 0.1% SDS] containing 10 μg/ml each of aprotinin and leupeptin. Lysis was achieved by gentle rotation at 4°C for 20 min. After centrifugation (14,000 × *g*, 10 min) to remove cell debris, lysates were incubated with 10 μg of antipeptide antibody and 30 μl of protein A/protein G (Calbiochem, La Jolla, CA) overnight. Beads were pelleted by centrifugation (14,000 × *g* 30 s), washed twice in lysis buffer containing 1 mM Na_3VO_4, and boiled in SDS sample buffer for 3 min. Proteins were resolved by 7.5% SDS-PAGE, transferred to nitrocellulose, and then probed with HER-2- or rat neu-specific monoclonal antibodies (Calbiochem). Protein transfer was monitored with prestained molecular weight standards (Bio-Rad). Immunoreactive bands were detected by enhanced chemiluminescence (Pierce) using horseradish peroxidase-conjugated goat antirabbit immunoglobulins.

Flow Cytometry. This procedure was adopted from that described by Hudziak *et al.* (13). Briefly, 5×10^5 SK-BR-3 or DHFR-G8 cells were incubated with either 2.5 μg of rabbit antipeptide antibodies or a 1:40 mouse sera dilution and HER-2-specific mouse monoclonal antibody Ab-2 and rat neu-specific monoclonal antibody Ab-4 (Calbiochem) were used as positive controls, and isotypic IgG was used as a negative control for 1 h at 4°C in 100 μl of PBS/1% FCS. The cells were washed twice in PBS and incubated with FITC-labeled secondary antibody (1:50 dilution) for 30 min at 4°C in 100 μl of PBS/1% FCS. The cells were washed twice, fixed in 2% formaldehyde, and analyzed by a Coulter ELITE flow cytometer (Coulter, Hialeah, FL). A total of 10,000 cells were counted for each sample, and final processing was performed. Debris, cell clusters, and dead cells were gated out by light scattered assessment before single-parameter histograms were drawn and smoothened.

Cell Proliferation Assay. SK-BR-3 and CAV-1 cells were plated at 5000 cells/well in V-bottomed plates with antipeptide antibodies at 10 μg/ml on day 0. On day 3, cells were pulsed with [^{3}H]thymidine (1 μCi/well) for 6 h and then placed in a −20°C freezer for 1 h. After thawing at room temperature, cells were harvested using a PHD cell harvester. Samples were incubated in 5 ml of Ready Safe liquid scintillation mixture (Beckman, Fullerton, CA), and radioactivity was determined by using a beta counter. Results are expressed as the percentage of inhibition [(untreated − treated)/untreated × 100] of triplicate samples.

ADCC Assay. PBMCs were isolated from heparinized whole blood obtained from normal human donors by density gradient sedimentation using Ficoll-Hypaque (Pharmacia Biotech, Piscataway, NJ). The purified PBMCs were washed twice with culture medium (RPMI 1640–1% FCS) and serially diluted into 96-well plates to give E:T ratios of 50:1, 25:1, 12.5:1, or 6.25:1. Protein A/G purified Her-2(628–647) peptide antibodies from immunized transgenic mice and the clinically applied HER-2 monoclonal antibody Trastuzumab (Genentech Inc, South San Francisco, CA) were added at 2 μg/well. Target cell lines (SK-BR-3 or BT-474) were labeled with 200 μCi of $Na^{51}CrO_4$ (New England Nuclear Life Science Products, Boston, MA) by incubating them for 45 min in a CO_2 humidified chamber at 37°C and washed three times in the cultured medium. A total of 0.1 ml of target cells (10^5/ml) was added per well for a final volume of 0.2 ml/well. Target cells were incubated with PBMCs in absence of antibodies to assess nonspecific lysis. The plates were incubated for 4 h at 37°C, and then the supernatants were harvested, and the radioactivity was determined using a gamma counter. The percentage of lysis or cytotoxicity was calculated as follows: Cytotoxicity (%) = $(A - B/C - B) \times 100$, where A represents ^{51}Cr (cpm) from test supernatants, B represents spontaneous release (^{51}Cr from target cells without antibody treatment), and C represents maximum release (^{51}Cr from target cells lysed with 5% Triton-X114). Each treatment was preformed in triplicate and averaged before calculating the percentage of lysis.

RESULTS

Chimeric HER-2 B-cell Epitope Constructs. Four of the 12 highest scoring (of the 144 analyzed) HER-2 ECD B-cell epitope sequences, amino acid sequences 115–136, 376–395, 410–429, and 628–647, were selected for evaluation (Table 1). Amino acid sequence alignment indicated that epitope 115–136 is highly variable between EGFR, HER-2, HER-3, and HER-4. Therefore, this sequence was hypothesized to have some unique function in HER-2 such as

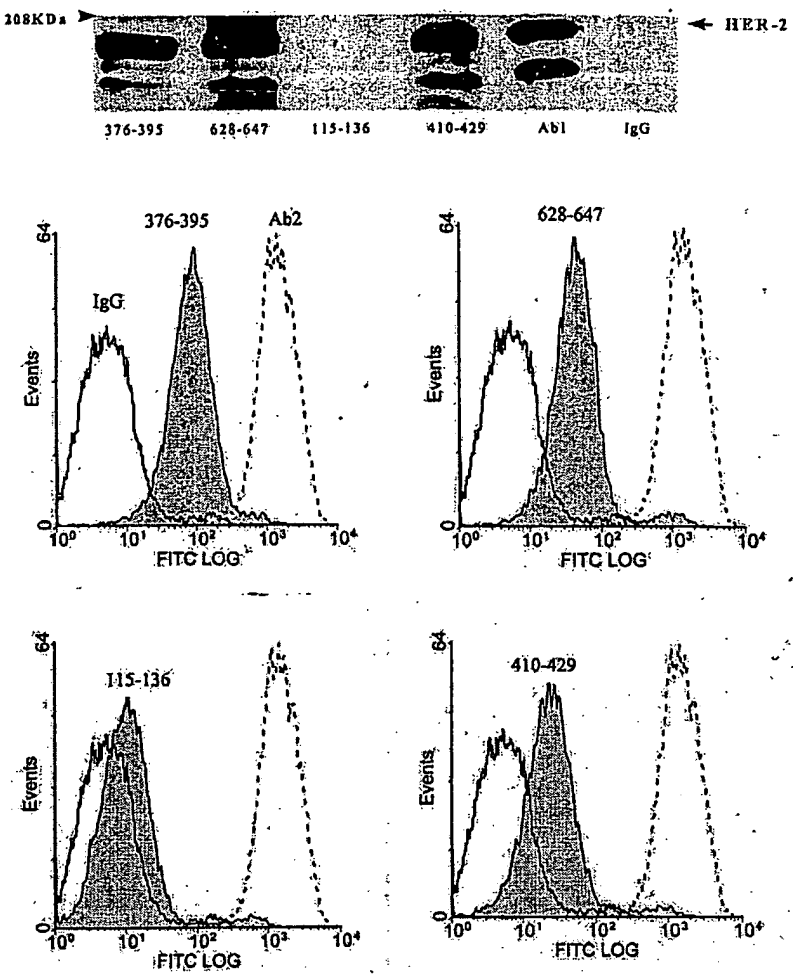

Fig. 2. Binding of peptide antibodies to the HER-2 receptor was determined by immunoprecipitation (*top*) and flow cytometry (*bottom*) using SB-BR-3 cells. IgG is an isotype antibody control. Ab-1 is a HER-2-specific monoclonal antibody used as a positive control for immunoprecipitation and Western blotting. Ab-2 is a mouse monoclonal antibody specific to the HER-2 ECD used as a positive control in flow cytometry.

ligand binding. Antibodies raised to this region were hypothesized to inhibit tumor growth by blocking HER-2 receptor signaling. Epitope 628–647 was chosen because of its proximity to the cell membrane. Antibodies binding to the juxtamembrane region were hypothesized to cause receptor aggregation and perturb the cell membrane more effectively, leading to HER-2 receptor endocytosis and degradation. Epitope sequences 376–395 and 410–426 were chosen because of their relative immunogenic potential, based on our predictive rankings. Neither of these two sequences contained cysteines or potential *N*-linked glycosylation sites, and these two sequences were predicted to form one secondary structural element, either an α-helix (sequence 376–395) or a β-sheet (sequence 410–429). HER-2 sequences 115–136, 376–395, and 410–429 were synthesized with the promiscuous T-helper cell epitope, MVF, at the COOH terminus, and sequence 628–647 was synthesized with MVF at the NH_2 terminus. The orientation of the T-helper cell epitope was chosen based on sequence-dependent difficulties for assembly of the peptide. However, the orientation of MVF does not affect the immunogenicity of the peptide constructs (46). These chimeric peptides incorporate a 4-residue linker (GPSL), in which glycine and proline in the linker potentiate a β turn in the oligopeptide, whereas serine in that position will favor hydrogen bonds with the free NH of the backbone. Leucine in the sequence was chosen because its side chain in that position is completely buried in the hydrophobic core and must be hydrophobic. The flexible nature of the linker allows for independent folding of the T-helper cell and B-cell epitopes (43, 46). HER-2 sequence 628–647 contains three cysteines whose disulfide bond pairing was unknown. Cys-634 and Cys-642 were hypothesized to form a suitable disulfide bridge, based on their proximity and predicted secondary structure. Thus, Cys-630 was mutated to glycine because the relatively small size of the R group of glycine causes minimal steric hindrance to formation of predicted β-sheet structure. Sequences 115–136 and 628–647 have potential *N*-linked glycosylation sites 124-NNTT-127 and 629-NCTH-632 respectively; however, the latter site is a poor sugar acceptor due to steric hindrance caused by the propensity of cysteine to form disulfide bonds (47). The crude peptides were purified by reverse-phase high-performance liquid chromatography and were >95% pure before immunization. The identity of the peptides was confirmed by mass spectrometry. The amino acid sequences, predicted secondary structures, posttranslational modifications, and the molecular weights of the MVF-conjugated HER-2 peptide constructs are indicated in Table 1.

Immunogenicity of Chimeric HER-2 B-cell Epitope Peptides in Outbred Rabbits. The HER-2 oligopeptides were highly immunogenic, as evidenced by antibody titers of over 100,000 (Fig. 1). HER-2(115–136) MVF elicited immediate and high antibody titers 1 week after the first booster in one of the two rabbits; however, the antibody response to this construct rose slowly in the other rabbit to high titers by 2 weeks after the second booster. The HER-2(376–395) MVF immune response was characterized by a slightly longer lag phase with an eventual rise in antibody titers to maximal levels after the tertiary boost. The antibody response to HER-2(410–29) MVF

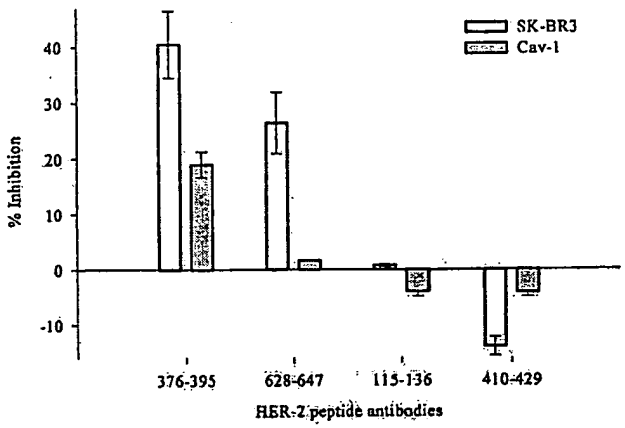

Fig. 3. Growth-inhibitory effects of peptide antibodies on SK-BR-3 and CAV-1 cells were determined by standard [^{3}H]thymidine proliferation assay. Results are expressed as the percentage of inhibition [untreated − treated/untreated × 100] of averaged triplicate samples. SDs are indicated by *error bars*.

was relatively low in both rabbits with maximum titers approaching 30,000 within 2 weeks after the tertiary boost. MVF HER-2(628–647) produced the most immediate and vigorous response, with exceptionally high titers of over 250,000 that remained at maximal levels in both rabbits through 4 weeks after tertiary boost. The polyclonal IgG sera did not cross-react with the MVF T-cell sequence.

Binding of Peptide Antibodies to Native HER-2 Receptor. Peptide-based vaccines will be effective only if the antibodies elicited by peptide immunogens bind the HER-2 receptor. Three different methods were used to test the binding of antipeptide antibodies to the native protein. First, the capacity of HER-2 peptide antibodies to immunoprecipitate HER-2 protein from the lysates of SK-BR-3 cells, a human breast cancer cell line overexpressing HER-2, was assessed. All of the peptide antibodies, except antibodies to HER-2(115–136) MVF, efficiently immunoprecipitated the native receptor from SK-BR-3 cells (Fig. 2). The lower band in the doublet is probably an isoform or underglycosylated counterpart due to high-level expression of HER-2 in SK-BR3 cells and has been observed by others (13, 34). Second, binding of the peptide antibodies to the intact HER-2 receptor was determined by immunofluorescence staining of single cell suspension of SK-BR-3 cells. Antibodies generated against HER-2(376–395) and HER-2(628–647) bound the receptor well within 1 log of HER-2-specific mouse monoclonal antibody Ab-2 (Fig. 2). However, antibodies to HER-2(410–429) showed weak binding and might be related to low titers elicited by rabbits against this immunogen (Fig. 1), whereas antibodies to HER-2(115–136) did not bind the receptor, confirming the immunoprecipitation results. Third, the binding of the peptide antibodies to a recombinant glycosylated HER-2 ECD was determined by an indirect ELISA. The pattern of reactivity of peptide antibodies with the protein paralleled that observed with immunoprecipitation and immunofluorescence staining (data not shown).

Effect on Breast Cancer Cell Proliferation. The effect of the peptide antibodies on tumor growth was examined *in vitro* with a standard [^{3}H]thymidine proliferation assay using HER-2-overexpressing SK-BR-3 cells and CAV-1, a colon cancer cell line that expresses low levels of HER-2 as a control. Antibodies elicited by HER-2(376–395) MVF and MVF HER-2(628–647) were able to reduce the proliferation of SK-BR-3 cells by about 40% and 30%, respectively, compared with untreated cells (Fig. 3). In contrast, slight increases in proliferation were observed with the antibodies elicited by HER-2(410–429) MVF. The antipeptide antibodies [with the exception of anti-HER-2(376–395) MVF] had minimal effects on CAV-1 cells, suggesting the possibility that peptide antibodies against MVF HER-2(628–647) selectively modulate the growth of HER-2-overexpressing tumor cell lines.

Effects of MVF-HER-2 Peptide Constructs in Rat neu Transgenic Mice. A transgenic mouse model (designated N202) developed by Guy *et al.* (48) that expresses mammary tumors similar to human breast cancer was used to test *in vivo* antitumor effects. Focal mammary tumors arise in at least 50% of the female transgenic mice around 28 weeks of age due to overexpression of the rat *neu* gene under the transcriptional control of the murine mammary tumor virus 3′ long terminal repeat. Three of the HER-2 peptide sequences (376–395, 410–429, and 628–647) have >80% homology to the analogous regions in rat neu (2). We examined whether the antibodies raised against the HER-2 peptides were capable of recognizing the rat neu receptor because there was a 20% amino acid sequence disparity. As depicted in Fig. 4, antibodies elicited with HER-2 sequences 115–136, 410–429, and 628–647 were able to immunoprecipitate the rat neu receptor from the *neu* gene-overexpressing DHFR-G8 fibroblast cell line.

Based on these results, female transgenic mice were immunized with HER-2(115–136) MVF, HER-2(410–429) MVF, and MVF HER-2(628–647), or MVF. MVF HER-2(628–647) elicited high-titered antibody responses against the immunogen of over 50,000 as early as 2 weeks after the second booster, and the antibody titers reached more than 250,000 after the third booster (Fig. 5). Antibodies against MVF HER-2(628–647) also reacted with recombinant HER-2 ECD with titers over 10,000 (Fig. 5) and the intact HER-2 and rat neu receptors of cells (data not shown). The transgenic mice did not mount appreciable antibody responses against immunogens HER-2(115–136) MVF and HER-2(410–429) MVF. Antibody titers against both these immunogens were below 4000 even 6 weeks after the fourth booster (data not shown).

By 48 weeks of age, all of the transgenic mice immunized with MVF emulsion, HER-2(115–136) MVF and HER-2(410–429) MVF, developed tumors of at least 10 millimeters in size. Most notably, in correlation with the *in vitro* inhibition of tumor cell proliferation (Fig. 3), 83% (five of six) transgenic mice immunized with MVF HER-2(628–647) were completely free of tumors (Fig. 6). MVF HER-2(628–647)-vaccinated mice showed a significantly longer tumor-free interval compared with mice immunized with MVF emulsion ($P = 0.0025$). No tumors were detectable in these mice during the 52 weeks of observation. Although there was a delay in the onset of tumors in the one mouse immunized with MVF HER-2(628–647) compared to other groups there was no significant difference in the kinetics of tumor growth after their occurrence (data not shown).

Fig. 4. Cross-reactivity of HER-2 peptide antibodies with rat neu receptor was determined by immunoprecipitation from the *neu* gene-overexpressing cell line DHFR-G8. Ab-4 is a monoclonal antibody specific to rat neu, and IgG is an isotype antibody control. The protein bands migrating just below the M_r 208,000 marker are shown by Western blotting with Ab-1, which also recognizes rat neu.

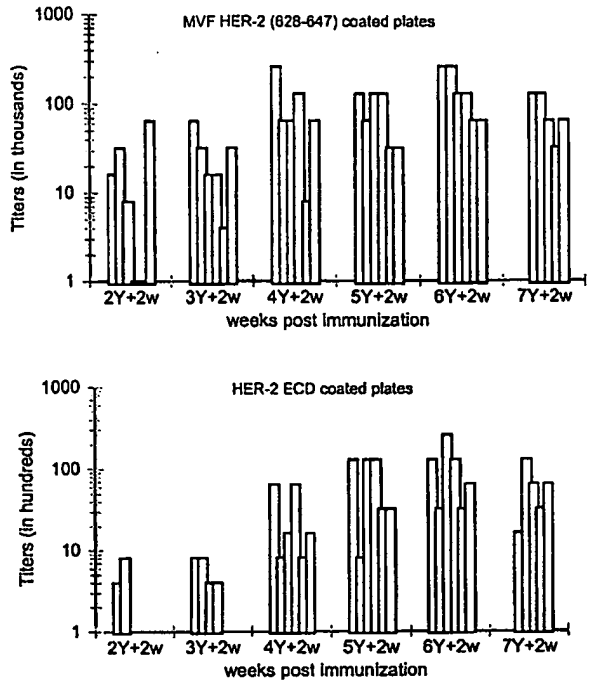

Fig. 5. Immune responses to MVF HER-2(628–647) in six transgenic mice (represented by individual bars) were determined by titering the sera against the corresponding immunogen (*top*) and glycosylated recombinant HER-2 ECD (*bottom*) by an indirect ELISA.

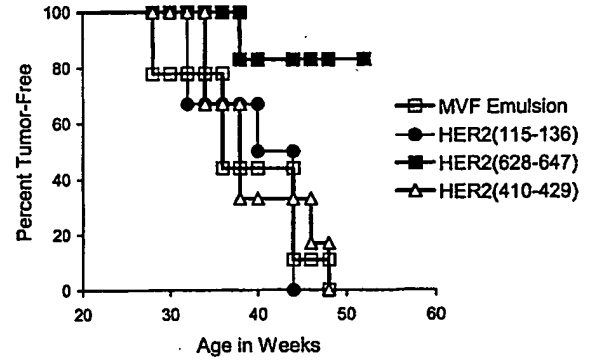

Fig. 6. Immunoprotective effects of HER-2 peptide epitopes on spontaneous tumor development in transgenic mice were determined by immunizing groups of six to nine mice with indicated peptides at 4 weeks of age and boosting every 4 or 8 weeks as described in "Materials and Methods." Tumor volumes were calculated as (length × width^2/2). The time to tumor development was analyzed using Kaplan-Meier survival analysis with log-rank comparisons of individual curves (62, 63).

Antibody-mediated Cytotoxicity of Breast Tumor Cell lines. We found IgG1 (58%) and IgG2 (35%) to be the major isotypes in the transgenic mouse sera elicited by MVF HER-2(628–647). HER-2 monoclonal antibodies representing these two isotypes were shown to be capable of mediating ADCC in conjunction with human PBMCs (22, 29). In an attempt to explore the molecular mechanism of tumor growth inhibition by the HER-2(628–647) peptide antibody, we tested its potential to recruit PBMCs to lyse HER-2-overexpressing mammary tumor cell lines in an ADCC assay. Peptide antibodies elicited in transgenic mice by HER-2(628–647) invoked lysis of two different human breast tumor cell lines, SK-BR-3 and BT-474, expressing high levels of HER-2 (27) in presence of human PBMCs, similar to the clinically applied HER-2 monoclonal antibody, Trastuzumab (Fig. 7).

DISCUSSION

The use of peptide immunogens in humans is considered problematic because they have been historically considered to be weak immunogens. Antibodies elicited in animals by immunization with synthetic peptides have generally been shown to have low affinity to the native protein, partly because antibody recognition sites are usually of the conformational type, and the peptide immunogens lacked defined structure in solution. Peptides must mimic the native conformation of the protein for their respective antibodies to bind target antigens with an affinity high enough to be biologically significant. The genetically restricted stimulatory activity of peptides is also a major obstacle to developing vaccine approaches for use in an outbred human population (49). Covalent conjugation of B-cell epitope peptides to large carrier molecules is sometimes used to address this problem but often results in hypersensitivity, conformational changes, appearance of undefined structures, loss of epitopes, inappropriate presentation of epitopes, and batch-to-batch conjugate variability. We have addressed several of these issues in our approach to subunit peptide vaccine design. Our strategy involved *de novo* design of topographic determinants that focused on preserving the native protein sequence while introducing a minimal number of rational point mutations to facilitate folding of the peptide into a stable conformation that mimics the native protein structure (50, 51). We have examined the effectiveness of incorporating promiscuous T-helper epitopes derived from nonhuman molecules into these constructs to overcome human MHC genetic polymorphism (41). Our previous work in a variety of model systems has demonstrated that this approach can elicit high-titered antibodies that recognize native protein in an outbred population (37–42).

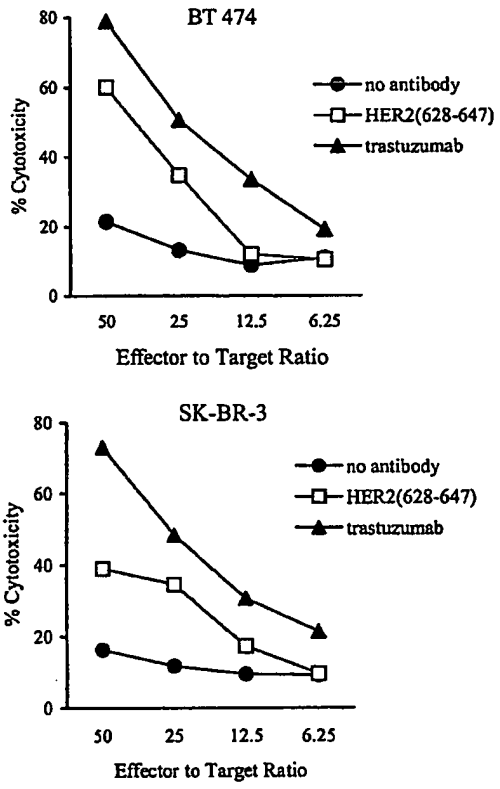

Fig. 7. HER-2(628–647) peptide antibody-mediated ADCC of ^{51}Cr-labeled mammary tumor target cell lines BT-474 (*top*) and SK-BR-3 (*bottom*) was assayed in presence of human PBMCs. The percentage of cytotoxicity was calculated as described in "Materials and Methods." SDs were less than 10% of the maximum individual values. Less than 2% lysis was observed when target cells were incubated with normal mouse immunoglobulin or antibodies alone without effector cells.

The antitumor activity of HER-2 monoclonal antibodies, some of which recognize denatured protein (22, 52, 53), prompted us to test the efficacy of antibodies raised against peptide immunogens from HER-2. In contrast to peptide vaccine approaches that have focused on multiple T-cell epitopes derived from HER-2 and the generation of anti-HER-2 T-cell responses (34), we have focused on individual B-cell determinants and optimizing an antibody response that has the potential of interfering with the transforming activity of HER-2. We demonstrate that a conformationally optimized chimeric B-cell peptide immunogen that incorporates a promiscuous T-helper epitope elicits high-titered antibodies to HER-2 in both outbred rabbits and inbred transgenic mice. Antibodies elicited by the chimeric peptide MVF HER-2(628–647) had antitumor activity *in vitro* and prevented tumor development *in vivo*. MVF HER-2(628–647) targets a membrane proximal region of the HER-2 ECD, which may be important in the antitumor activity observed with this immunogen. The epitope recognized by the clinically applied HER-2 monoclonal antibody Trastuzamab is reported to be located in the membrane proximal region of the ECD, amino acids 529–627 (54). This antibody has been shown to induce HER-2 receptor degradation (13), inhibit receptor cross-talk (55), and mediate ADCC (27, 54). It is possible that the peptide antibodies elicited by HER-2(628–647) may inhibit tumor growth by these mechanisms as represented by their capacity to mediate ADCC. Optimal cysteine disulfide pairings in the sequence 628–647 have been shown to be critical for transforming activity of HER-2/neu. Disruption of disulfide bonds in this region has been shown to promote ligand-independent receptor homodimerization and early tumor development (56, 57).

Although HER-2(376–395) MVF peptide elicited native receptor-specific antibodies and inhibited tumor cell proliferation in tissue culture, we were unable to evaluate the immunoprotective capacity of this construct in neu transgenic mice because these peptide antibodies failed to cross-react with rat neu receptor. Not all antibodies elicited demonstrated antiproliferative activity. An increase in tumor proliferation was observed *in vitro* with the antibodies elicited by HER-2(410–429) MVF. Monoclonal antibodies to different HER-2 epitopes have demonstrated differential effects. Some bind and display no activity, whereas others stimulate or inhibit tumor growth (22, 52, 53, 58). The predicted B-cell epitopes were not equally immunogenic nor did they equally elicit antibody that bound native HER-2 protein. HER-2(115–136) MVF and HER-2(410–429) MVF were poorly immunogenic in transgenic mice. Strong immune responses to HER-2(376–395) prompted us to evaluate the adjacent epitope HER-2(410–429), although it had the lowest predicted score of the final 12 epitope selections. This low score may explain its poor immunogenicity. Glycosylation is shown to play a decisive role in the immunogenicity of tumor-associated antigens (59) with effects on both the structure of the B-cell determinants and their ability to bind antibodies (60, 61). The inability of HER-2(115–136) MVF peptide antibodies to recognize the receptor might be due to the absence of sugars at the predicted *N*-linked glycosylation site (NNTT) in the synthetic peptide in contrast to other immunogens. This observation is supported by the fact that the antibodies raised against HER-2(115–136) MVF peptide were able to immunoprecipitate the rat neu receptor (Fig. 4). It is interesting to note that rat neu receptor does not harbor a *N*-linked glycosylation site in the analogous region (2).

It is not clear why only a minority of patients with tumors that overexpress HER-2 have manifested objective clinical responses with passive immunotherapy with Trastuzumab (19, 21). Tissue distribution and levels and the inherent immunogenicity of monoclonal antibodies, even humanized constructs, are a few of the major constraints associated with passive therapy. It should also be noted that antitumor activity of HER-2 antibodies in preclinical studies has been only partial and is cytostatic in nature. Because of this, prolonged therapy is necessary. The cost and antigenicity of monoclonal antibody also pose major obstacles for this application. In contrast, synthetic subunit peptide vaccines are attractive when targeting proteins such as HER-2. Oligopeptide vaccines are cost-effective to produce and are more readily characterized during their manufacture. More importantly, subunit peptide vaccines can focus immune responses to biologically active epitopes. The capacity to narrowly focus the immune response is of particular relevance to HER-2, where interaction of the antibody with specific sites has the potential of stimulating growth. In contrast to passive therapy, the continuous availability of tumor-targeting antibodies can be ensured at low cost. Here we report a B-cell epitope vaccine capable of eliciting HER-2-specific antibodies in an outbred population with a potential to suppress the development of HER-2-overexpressing cancers. A National Cancer Institute-supported Phase I clinical trial to evaluate both the immunogenicity and toxicity of MVF HER-2(628–647) is currently under way at the Ohio State University Medical Center.

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REFERENCES

1. Coussens, L., Yang-Feng, T. L., Liao, Y. C., Chen, E., Gray, A., McGrath, J., Seeburg, P. H., Libermann, T. A., Schlessinger, J., Francke, U., Levinson, A., and Ullrich, A. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. Science (Washington DC), *230:* 1132–1139, 1985.
2. Yamamoto, T., Ikawa, S., Akiyama, T., Semba, K., Nomura, N., Miyajima, N., Saito, T., and Toyoshima, K. Similarity of protein encoded by the human c-erb-B-2 gene to epidermal growth factor receptor. Nature (Lond.), *319:* 230–234, 1986.
3. Tzahar, E., Pinkas-Kramarski, R., Moyer, J. D., Klapper, L. N., Alroy, I., Levkowitz, G., Shelly, M., Henis, S., Eisenstein, M., Ratzkin, B. J., Sela, M., Andrews, G. C., and Yarden, Y. Bivalence of EGF-like ligands drives the ErbB signaling network. EMBO J., *16:* 4938–4950, 1997.
4. Tzahar, E., and Yarden, Y. The ErbB-2/HER2 oncogenic receptor of adenocarcinomas: from orphanhood to multiple stromal ligands. Biochim. Biophys. Acta, *1377:* M25–M37, 1998.
5. Press, M. F., Cordon-Cardo, C., and Slamon, D. J. Expression of the HER-2/neu proto-oncogene in normal human adult and fetal tissues. Oncogene, *5:* 953–962, 1990.
6. Cirisano, F. D., and Karlan, B. Y. The role of the HER-2/neu oncogene in gynecologic cancers. J. Soc. Gynecol. Investig., *3:* 99–105, 1996.
7. Ross, J. S., and Fletcher, J. A. The HER-2/neu oncogene: prognostic factor, predictive factor and target for therapy. Semin. Cancer Biol., *9:* 125–138, 1999.
8. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A., and Press, M. F. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science (Washington DC), *244:* 707–712, 1989.
9. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science (Washington DC), *235:* 177–182, 1987.
10. Nicholson, R. I., McClelland, R. A., Gee, J. M., Manning, D. L., Cannon, P., Robertson, J. F., Ellis, I. O., and Blamey, R. W. Epidermal growth factor receptor expression in breast cancer: association with response to endocrine therapy. Breast Cancer Res. Treat., *29:* 117–125, 1994.
11. Alroy, I., and Yarden, Y. The ErbB signaling network in embryogenesis and oncogenesis: signal diversification through combinatorial ligand-receptor interactions. FEBS Lett., *410:* 83–86, 1997.
12. Lonardo, F., Di Marco, E., King, C. R., Pierce, J. H., Segatto, O., Aaronson, S. A., and Di Fiore, P. P. The normal erbB-2 product is an atypical receptor-like tyrosine kinase with constitutive activity in the absence of ligand. New Biol., *2:* 992–1003, 1990.
13. Hudziak, R. M., Lewis, G. D., Winget, M., Fendly, B. M., Shepard, H. M., and Ullrich, A. p185HER2 monoclonal antibody has antiproliferative effects *in vitro* and sensitizes human breast tumor cells to tumor necrosis factor. Mol. Cell. Biol., *9:* 1165–1172, 1989.
14. Tagliabue, E., Centis, F., Campiglio, M., Mastroianni, A., Martignone, S., Pellegrini, R., Casalini, P., Lanzi, C., Menard, S., and Colnaghi, M. I. Selection of monoclonal antibodies which induce internalization and phosphorylation of p185HER2 and growth inhibition of cells with HER2/NEU gene amplification. Int. J. Cancer, *47:* 933–937, 1991.
15. Drebin, J. A., Link, V. C., Weinberg, R. A., and Greene, M. I. Inhibition of tumor growth by a monoclonal antibody reactive with an oncogene-encoded tumor antigen. Proc. Natl. Acad. Sci. USA, *83:* 9129–9133, 1986.

16. Drebin, J. A., Link, V. C., and Greene, M. I. Monoclonal antibodies reactive with distinct domains of the neu oncogene-encoded p185 molecule exert synergistic anti-tumor effects *in vivo*. Oncogene, *2:* 273–277, 1988.
17. Drebin, J. A., Link, V. C., and Greene, M. I. Monoclonal antibodies specific for the neu oncogene product directly mediate anti-tumor effects *in vivo*. Oncogene, *2:* 387–394, 1988.
18. Katsumata, M., Okudaira, T., Samanta, A., Clark, D. P., Drebin, J. A., Jolicoeur, P., and Greene, M. I. Prevention of breast tumour development *in vivo* by downregulation of the p185neu receptor. Nat. Med., *1:* 644–648, 1995.
19. Baselga, J., Tripathy, D., Mendelsohn, J., Baughman, S., Benz, C. C., Dantis, L., Sklarin, N. T., Seidman, A. D., Hudis, C. A., Moore, J., Rosen, P. P., Twaddell, T., Henderson, I. C., and Norton, L. Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. J. Clin. Oncol., *14:* 737–744, 1996.
20. Cobleigh, M. A., Vogel, C. L., Tripathy, D., Robert, N. J., Scholl, S., Fehrenbacher, L., Wolter, J. M., Paton, V., Shak, S., Lieberman, G., and Slamon, D. J. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. J. Clin. Oncol., *17:* 2639–2648, 1999.
21. Pegram, M. D., Lipton, A., Hayes, D. F., Weber, B. L., Baselga, J. M., Tripathy, D., Baly, D., Baughman, S. A., Twaddell, T., Glaspy, J. A., and Slamon, D. J. Phase II study of receptor-enhanced chemosensitivity using recombinant humanized anti-p185HER2/neu monoclonal antibody plus cisplatin in patients with HER2/neu-overexpressing metastatic breast cancer refractory to chemotherapy treatment. J. Clin. Oncol., *16:* 2659–2671, 1998.
22. Stancovski, I., Hurwitz, E., Leitner, O., Ullrich, A., Yarden, Y., and Sela, M. Mechanistic aspects of the opposing effects of monoclonal antibodies to the ERBB2 receptor on tumor growth. Proc. Natl. Acad. Sci. USA, *88:* 8691–8695, 1991.
23. Maier, L. A., Xu, F. J., Hester, S., Boyer, C. M., McKenzie, S., Bruskin, A. M., Argon, Y., and Bast, R. C., Jr. Requirements for the internalization of a murine monoclonal antibody directed against the HER-2/neu gene product c-erbB-2. Cancer Res., *51:* 5361–5369, 1991.
24. Klapper, L. N., Vaisman, N., Hurwitz, E., Pinkas-Kramarski, R., Yarden, Y., and Sela, M. A subclass of tumor-inhibitory monoclonal antibodies to ErbB-2/HER2 blocks crosstalk with growth factor receptors. Oncogene, *14:* 2099–2109, 1997.
25. Ghetie, M. A., Podar, E. M., Ilgen, A., Gordon, B. E., Uhr, J. W., and Vitetta, E. S. Homodimerization of tumor-reactive monoclonal antibodies markedly increases their ability to induce growth arrest or apoptosis of tumor cells. Proc. Natl. Acad. Sci. USA, *94:* 7509–7514, 1997.
26. Kita, Y., Tseng, J., Horan, T., Wen, J., Philo, J., Chang, D., Ratzkin, B., Pacifici, R., Brankow, D., Hu, S., Luo, Y., Wen, D., Arakawa, T., and Nicolson, M. ErbB receptor activation, cell morphology changes, and apoptosis induced by anti-Her2 monoclonal antibodies. Biochem. Biophys. Res. Commun., *226:* 59–69, 1996.
27. Lewis, G. D., Figari, I., Fendly, B., Wong, W. L., Carter, P., Gorman, C., and Shepard, H. M. Differential responses of human tumor cell lines to anti-p185HER2 monoclonal antibodies. Cancer Immunol. Immunother., *37:* 255–263, 1993.
28. Jurianz, K., Maslak, S., Garcia-Schuler, H., Fishelson, Z., and Kirschfink, M. Neutralization of complement regulatory proteins augments lysis of breast carcinoma cells targeted with rhumAb anti-HER2. Immunopharmacology, *42:* 209–218, 1999.
29. Stockmeyer, B., Valerius, T., Repp, R., Heijnen, I. A., Buhring, H. J., Deo, Y. M., Kalden, J. R., Gramatzki, M., and van de Winkel, J. G. Preclinical studies with Fc(γ)R bispecific antibodies and granulocyte colony-stimulating factor-primed neutrophils as effector cells against HER-2/neu-overexpressing breast cancer. Cancer Res., *57:* 696–701, 1997.
30. Bernards, R., Destree, A., McKenzie, S., Gordon, E., Weinberg, R. A., and Panicali, D. Effective tumor immunotherapy directed against an oncogene-encoded product using a vaccinia virus vector. Proc. Natl. Acad. Sci. USA, *84:* 6854–6858, 1987.
31. Gu, X. G., Schmitt, M., Hiasa, A., Nagata, Y., Ikeda, H., Sasaki, Y., Akiyoshi, K., Sunamoto, J., Nakamura, H., Kuribayashi, K., and Shiku, H. A novel hydrophobized polysaccharide/oncoprotein complex vaccine induces *in vitro* and *in vivo* cellular and humoral immune responses against HER2-expressing murine sarcomas. Cancer Res., *58:* 3385–3390, 1998.
32. Esserman, L. J., Lopez, T., Montes, R., Bald, L. N., Fendly, B. M., and Campbell, M. J. Vaccination with the extracellular domain of p185neu prevents mammary tumor development in neu transgenic mice. Cancer Immunol. Immunother., *47:* 337–342, 1999.
33. Cefai, D., Morrison, B. W., Sckell, A., Favre, L., Balli, M., Leunig, M., and Gimmi, C. D. Targeting HER-2/neu for active-specific immunotherapy in a mouse model of spontaneous breast cancer. Int. J. Cancer, *83:* 393–400, 1999.
34. Disis, M. L., Gralow, J. R., Bernhard, H., Hand, S. L., Rubin, W. D., and Cheever, M. A. Peptide-based, but not whole protein, vaccines elicit immunity to HER-2/neu, oncogenic self-protein. J. Immunol., *156:* 3151–3158, 1996.
35. Disis, M. L., Grabstein, K. H., Sleath, P. R., and Cheever, M. A. Generation of immunity to the HER-2/neu oncogenic protein in patients with breast and ovarian cancer using a peptide-based vaccine. Clin. Cancer Res., *5:* 1289–1297, 1999.
36. Partidos, C. D., and Steward, M. W. Prediction and identification of a T cell epitope in the fusion protein of measles virus immunodominant in mice and humans. J. Gen. Virol., *71:* 2099–2105, 1990.
37. Kaumaya, P. T. P., Feng, N., Kobs-Conrad, S., Seo, Y. H., Vanbuskirk, A. M., and Sheridan, J. F. Immunogenicity and antigenicity of a promiscuous T cell epitope and a topographic B cell determinant of the protein antigen LDH-C4. *In:* J. A. Smith, J. E. Rivier (eds.), Peptides: Chemistry and Biology, pp. 883–885. Leiden: ESCOM, 1992.
38. Seo, Y. H., Kobs-Conrad, S., and Kaumaya, P. T. P. Promiscuous T cell epitopes of tetanus toxin, and measles virus enhances immune responses to B cell epitopes of the protein antigen LDH-C4. *In:* C. H. Schneider and A. N. Eberle (eds.), Peptides, p. 866–868. Leiden: ESCOM, 1992.
39. Bakaletz, L. O., Leake, E. R., Billy, J. M., and Kaumaya, P. T. Relative immunogenicity and efficacy of two synthetic chimeric peptides of fimbrin as vaccinogens against nasopharyngeal colonization by nontypeable *Haemophilus influenzae* in the chinchilla. Vaccine, *15:* 955–961, 1997.
40. Srinivasan, M., Domanico, S. Z., Kaumaya, P. T., and Pierce, S. K. Peptides of 23 residues or greater are required to stimulate a high affinity class II-restricted T cell response. Eur. J. Immunol., *23:* 1011–1016, 1993.
41. Kaumaya, P. T., Kobs-Conrad, S., Seo, Y. H., Lee, H., Van Buskirk, A. M., Feng, N., Sheridan, J. F., and Stevens, V. Peptide vaccines incorporating a "promiscuous" T-cell epitope bypass certain haplotype restricted immune responses and provide broad spectrum immunogenicity. J. Mol. Recognit., *6:* 81–94, 1993.
42. Lairmore, M. D., DiGeorge, A. M., Conrad, S. F., Trevino, A. V., Lal, R. B., and Kaumaya, P. T. Human T-lymphotropic virus type I peptides in chimeric and multivalent constructs with promiscuous T-cell epitopes enhance immunogenicity and overcome genetic restriction. J. Virol., *69:* 6077–6089, 1995.
43. Kaumaya, P. T. P., Kobs-Conrad, S., DiGeorge, A. M., and Stevens, V. *De novo* engineering of protein immunogenic and antigenic determinants. *In:* G. M. Anantharamaiah and C. Basava (eds.), Peptides, Design, Synthesis & Biological Activity. pp. 133–164. Boston: Birkhauser, 1994.
44. Kobs-Conrad, S., Lee, H., DiGeorge, A. M., and Kaumaya, P. T. P. Engineered topographic determinants with $\alpha\beta$, $\beta\alpha\beta$, and $\beta\alpha\beta\alpha$ topologies show high affinity binding to native protein antigen LDH-C4. J. Biol. Chem., *268:* 25285–25295, 1993.
45. Jones, W. R., Bradley, J., Judd, S. J., Denholm, E. H., Ing, R. M., Mueller, U. W., Powell, J., Griffin, P. D., and Stevens, V. C. Phase I clinical trial of a World Health Organisation birth control vaccine. Lancet, *1:* 1295–1298, 1988.
46. Kaumaya, P. T. P. Synthetic peptide vaccines: dream or reality. *In:* C. H. Schneider (ed.), Peptides in Immunology, pp. 117–148. United Kingdom: Wiley and Sons, Ltd., 1996.
47. Kasturi, L., Chen, H., and Shakin-Eshleman, S. H. Regulation of N-linked core glycosylation: use of a site-directed mutagenesis approach to identify Asn-Xaa-Ser/Thr sequons that are poor oligosaccharide acceptors. Biochem. J., *323:* 415–419, 1997.
48. Guy, C. T., Webster, M. A., Schaller, M., Parsons, T. J., Cardiff, R. D., and Muller, W. J. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc. Natl. Acad. Sci. USA, *89:* 10578–10582, 1992.
49. Deulofeut, H., Iglesias, A., Mikael, N., Bing, D. H., Awdeh, Z., Yunis, J., Marcus-Bagley, D., Kruskall, M. S., Alper, C. A., and Yunis, E. J. Cellular recognition and HLA restriction of a midsequence HBsAg peptide in hepatitis B vaccinated individuals. Mol. Immunol., *30:* 941–948, 1993.
50. Kobs-Conrad, S., Lee, H., DiGeorge, A. M., and Kaumaya, P. T. Engineered topographic determinants with $\alpha\beta$, $\beta\alpha\beta$, and $\beta\alpha\beta\alpha$ topologies show high affinity binding to native protein antigen (lactate dehydrogenase-C4). J. Biol. Chem., *268:* 25285–25295, 1993.
51. Kaumaya, P. T., Berndt, K. D., Heidorn, D. B., Trewhella, J., Kezdy, F. J., and Goldberg, E. Synthesis and biophysical characterization of engineered topographic immunogenic determinants with $\alpha\alpha$ topology. Biochemistry, *29:* 13–23, 1990.
52. Boyer, C. M., Pusztai, L., Wiener, J. R., Xu, F. J., Dean, G. S., Bast, B. S., O'Briant, K. C., Greenwald, M., DeSombre, K. A., and Bast, R. C., Jr. Relative cytotoxic activity of immunotoxins reactive with different epitopes on the extracellular domain of the c-erbB-2 (HER-2/neu) gene product p185. Int. J. Cancer, *82:* 525–531, 1999.
53. Xu, F., Lupu, R., Rodriguez, G. C., Whitaker, R. S., Boente, M. P., Berchuck, A., Yu, Y., DeSombre, K. A., Boyer, C. M., and Bast, R. C., Jr. Antibody-induced growth inhibition is mediated through immunochemically and functionally distinct epitopes on the extracellular domain of the c-erbB-2 (HER-2/neu) gene product p185. Int. J. Cancer, *53:* 401–408, 1993.
54. Sliwkowski, M. X., Lofgren, J. A., Lewis, G. D., Hotaling, T. E., Fendly, B. M., and Fox, J. A. Nonclinical studies addressing the mechanism of action of trastuzumab (Herceptin). Semin. Oncol., *26:* 60–70, 1999.
55. Lewis, G. D., Lofgren, J. A., McMurtrey, A. E., Nuijens, A., Fendly, B. M., Bauer, K. D., and Sliwkowski, M. X. Growth regulation of human breast and ovarian tumor cells by heregulin: evidence for the requirement of ErbB2 as a critical component in mediating heregulin responsiveness. Cancer Res., *56:* 1457–1465, 1996.
56. Siegel, P. M., Ryan, E. D., Cardiff, R. D., and Muller, W. J. Elevated expression of activated forms of Neu/ErbB-2 and ErbB-3 are involved in the induction of mammary tumors in transgenic mice: implications for human breast cancer. EMBO J., *18:* 2149–2164, 1999.
57. Siegel, P. M., and Muller, W. J. Mutations affecting conserved cysteine residues within the extracellular domain of Neu promote receptor dimerization and activation. Proc. Natl. Acad. Sci. USA, *93:* 8878–8883, 1996.
58. Hurwitz, E., Stancovski, I., Sela, M., and Yarden, Y. Suppression and promotion of tumor growth by monoclonal antibodies to ErbB-2 differentially correlate with cellular uptake. Proc. Natl. Acad. Sci. USA, *92:* 3353–3357, 1995.
59. Ciborowski, P., and Finn, O. J. Recombinant epithelial cell mucin (MUC-1) expressed in baculovirus resembles antigenically tumor associated mucin, target for cancer immunotherapy. Biomed. Pept. Proteins and Nucleic Acids, *1:* 193–198, 1995.
60. Huang, X., Barchi, J. J. J., Lung, F. D., Roller, P. P., Nara, P. L., Muschik, J., and Garrity, R. R. Glycosylation affects both the three-dimensional structure and antibody binding properties of the HIV-1IIIB GP120 peptide RP135. Biochemistry, *36:* 10846–10856, 1997.
61. Trkola, A., Purtscher, M., Muster, T., Ballaun, C., Buchacher, A., Sullivan, N., Srinivasan, K., Sodroski, J., Moore, J. P., and Katinger, H. Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. J. Virol., *70:* 1100–1108, 1996.
62. Kaplan, E. L., and Meier, P. Nonparametric estimation from incomplete observations. J. Am. Stat. Assoc., *53:* 457–481, 1958.
63. Mantel, N. Evaluation of survival data and two new rank order statistics arising in its consideration. Cancer Chemother. Rep., *50:* 163–170, 1966.

Single Chain Ig/γ Gene-Redirected Human T Lymphocytes Produce Cytokines, Specifically Lyse Tumor Cells, and Recycle Lytic Capacity[1]

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To enable construction of CTL with known predefined Ab specificity for adoptive immunotherapy, we constructed a chimeric scFv/γ gene composed of the variable regions of a mAb joined to the Fc(ϵ)RI signaling receptor γ-chain of mast cells. Introduction of this chimeric receptor into CTL rendered these lymphocytes specific for renal cell carcinoma. This approach combines the specificity of tumor-selective Abs with the efficacy of CTL to destroy tumor cells. We not only demonstrated that the transduced CTL functionally express the scFv/γ receptor for a prolonged period of time (4.5 mo of in vitro culture), but also showed high levels of Ab-dictated lysis of renal cell carcinoma similar to that of normal CTL, and importantly, we demonstrated that these CTL can recycle their lytic activity. Moreover, these scFv/γ-expressing T lymphocytes produce cytokines upon stimulation with the relevant target cell. These results together with the donor independence of our gene transduction protocol demonstrate the feasibility of redirecting T lymphocytes for cancer treatment. *The Journal of Immunology,* **1996, 157: 836–843.**

Adoptive cellular immunotherapy in cancer treatment refers to the transfer of cultured immune cells with antitumor reactivity into patients. Lymphokine-activated killer cells and tumor-infiltrating lymphocytes have shown therapeutic responses in clinical trials, although these were observed in only a fraction of the patients treated (1, 2). Tumor-specific MHC-restricted CTL have been isolated, primarily specific for melanoma (3), but the availability of tumor-specific lymphocytes against more common types of cancer for adoptive therapy has been limited due to difficulties in generating these tumor-specific CTL. Broadening of the range of tumor specificity has been obtained by combining biologic response modifier (BRM)[3] production, migration/homing, as well as lytic capacity of T lymphocytes with the selectivity of tumor-recognizing mAbs. Bispecific mAbs (bsmAb), with one binding site directed against a tumor Ag and the other recognizing an activation molecule on the T lymphocyte, have been employed to redirect CTL in preclinical and clinical studies (4–8). However, the use of bsmAb for therapy may be hampered by the inaccessibility of solid tumors to Ab penetration (9). Moreover, bsmAb-redirected CTL retain the bispecific Ab for only limited periods of time (i.e., 48–96 h) due to their dissociation of the CTL surface (4, 10). In addition, bsmAb-redirected CTL lose signal transducing and, hence, lytic capacity following target cell recognition, lysis, and TCR/CD3 complex clustering (10).

To circumvent the limitations associated with bsmAb, we and others have adopted an approach in which T lymphocytes are grafted with a permanent Ab-dictated specificity (11, 12). Variable domains of mAb fused by a flexible linker sequence have been shown to display binding affinities and specificities similar to those of the natural mAb (13–15). Such single chain Abs (scFv) juxtaposed to a signal transducing molecule such as the Fc(ϵ)RI γ- or TCR ζ-chain have been functionally expressed in mouse T cell hybridomas or CTL (11, 12, 16, 17), tumor-infiltrating lymphocytes (18), and human $CD8^+$ T lymphocytes (19). Stimulation of the chimeric receptor with the relevant target cell results in T cell activation responses, including BRM production (16) and lysis of the target cell (11, 12, 17–19).

In this study we used an scFv/γ receptor derived from a renal cell carcinoma-selective mouse mAb G250 that is directed to a membrane-bound Ag present on >90% of primary tumors and >80% of metastases (20, 21). A chimeric gene was constructed composed of the variable domains of the G250 mAb and the γ-chain from the Fc(ϵ)RI receptor present on mast cells (22). Retroviral gene transfer was employed to successfully transduce the scFv/γ into activated T lymphocytes. The gene-transduced T lymphocytes stably express the receptor for >4 mo and specifically lyse renal cell carcinoma in an MHC-unrestricted manner. Continuing and advancing the work of others (11, 18), successful transduction was demonstrated in T lymphocytes derived from all donors tested, as shown by 1) scFv/γ-redirected lysis, 2) cytokine production by the gene-transduced T lymphocytes upon relevant target cell interaction, and 3) the fact that, like normal CTL (10, 19, 23, 24), the gene-transduced CTL can recycle their scFv/γ-dictated lytic activity, i.e., one CTL enters into multiple lytic cycles with the target cells.

Materials and Methods

Cells and antibodies

PBL from healthy donors were isolated by centrifugation through Ficoll-Isopaque (density = 1.077 g/cm^3; Pharmacia Fine Chemicals, Uppsala,

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[3] Abbreviations used in this paper: BRM, biologic response modifier; bsmAb, bispecific monoclonal antibody; RT-PCR, reverse transcription-polymerase chain reaction; GM-CSF, granulocyte-macrophage colony-stimulating factor; AK, activated kill.

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Sweden) and activated in culture flasks precoated for 3 h at 37°C with a 1/30 dilution of OKT3 culture supernatant at a density of 2×10^6 cells/ml for 3 days in Mix-Med culture medium (78% RPMI 1640 buffered with bicarbonate (2 g/l) and HEPES (25 mM), 20% AIM-V (Life Technologies, Paisley, U.K.), and 2% heat-inactivated human plasma supplemented with 360 IU/ml human rIL-2 (Eurocetus, Amsterdam, The Netherlands), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) (25). In addition, lymphocytes were activated in Mix-Med culture medium with 10 ng/ml OKT3 (Ortho Diagnostic System, Beerse, Belgium) at a density of 0.5×10^6 cells/ml without rIL-2. After activation, lymphocytes were washed twice and cultured in Mix-Med culture medium. The following cell lines were used as target cells in the cytotoxicity assays: renal cell carcinoma cell lines SK-RC-7 (kindly provided by S. Warnaar, Leiden, The Netherlands), SK-RC-52, SK-RC-1, SK-RC-59, and SK-RC-10 (kindly provided by E. Oosterwijk, Nijmegen, The Netherlands), and A75 (generated in our laboratory); melanoma cell line G43 (kindly provided by T. Boon, Brussels, Belgium); ovarian carcinoma cell line IGROV-1 (kindly provided by J. Bénard, Villejuif, France); Burkitt lymphoma-derived cell line Daudi; and erythromyeloid-derived cell line K562. The amphotropic packaging cell line PA317 (American Type Culture Collection, Rockville, MD) was cultured in DMEM 12501 (Life Technologies) and 10% bovine calf serum (HyClone, Logan, UT) supplemented with 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The mAbs used in cytotoxicity inhibition studies were the renal tumor-associated Ag (TAA)-specific G250 mAb (kindly provided by S. O. Warnaar, Centocor, Leiden, The Netherlands) and anti-HLA-A,B,C mAb (W6/32, Seralab, Sussex, U.K.).

Construction of scFv/γ genes

The genes encoding the V_H and the V_L domains of the G250 mouse mAb were isolated by anchored PCR (26) from cDNA prepared from G250 mAb-producing hybridoma cells, using anchored/anchored poly(C) primers (26) and a constant V_H (HB) or V_L (KA) primer. The V_H and V_L gene segments were cloned into the plasmid pGEM11, and the nucleotide sequence was determined using the dideoxy-mediated chain termination method (Pharmacia). To construct the scFv/γ chimeric receptor, we used a pBluescript vector containing the linker sequence 212 (14). The V_H gene segment containing the leader sequence was reamplified using primers introducing *Eco*RI and *Bam*HI restriction sites at the 5′ and 3′ ends, respectively. The V_L gene segment was reamplified without the leader sequence using primers introducing *Xba*I and *Bam*HI restriction sites at the 5′ and 3′ ends, respectively. The V_L gene 3′ primer consists of a constant light chain (C_L) gene sequence (18 nucleotides) and an extracellular ζ gene sequence (nucleotides 138–158) (27). The Fc(ε)RI γ-chain, containing the most 3′ 9-bp extracellular sequence in addition to transmembrane and intracellular sequences, was isolated from a human cDNA clone (22) using *Bam*HI and *Xho*I restriction sites at the 5′ and 3′ ends (kindly provided by Z. Eshhar, Rehovot, Israel). To construct the chimeric scFv/γ gene, V_H, V_L, and γ gene segments were cloned into the pBluescript vector. The chimeric scFv/γ gene was subsequently subcloned into the retroviral vector LXSN (28) containing the murine Moloney leukemia virus long terminal repeat and a neomycin resistance gene under the control of an SV40 promoter. The sequences of the primers used are: HB, CTC TAA GCT TGG CTC AAA CAC AGC GAC CTC GGA TAC AGT TGG TGC AGC; KA, CTC TTC TAG AGA GTC TCT CAG CTG GTA GGA TAC AGT TGG TGC AGC: V_H5', CGC TCG AGG AAT TCG CAC TGA ACA CAG ACC; V_H3', GCG CGG ATC CTG AGG AGA CGG TGA CTG A; V_L5', CTA GTC TAG AGA CAT TGT GAT GAC CGA G; and V_L3', CGC GCG GAT CCA GCA GGC CAA AGC TCT GGG ATA CAG TTG GTG CAG C.

Gene transduction and selection of gene-transduced lymphocytes

The LXSN retroviral vectors were electroporated into the amphotropic packaging cell line PA317 using a BTX electroporator (BTX, San Diego, CA) at 250 V and a capacitance of 750 μF. A stable amphotropic packaging line, PA317, was obtained after G418 selection. The amphotropic virus supernatants produced had a viral titer of 1×10^4 CFU/ml, determined on the basis of neomycin resistance of infected NIH-3T3 cells. To transduce the activated PBL with the G250 scFv/γ retroviral vector (L(scFv/γG250)SN), 2×10^6 lymphocytes were cocultivated for 72 h with a 70% confluent irradiated (25 Gy) monolayer of virus-producing cells in culture medium supplemented with 4 μg/ml polybrene (Sigma Chemical Co., St. Louis, MO) and 360 IU/ml rIL-2. Subsequently, the gene-transduced PBL population was selected for 4 days in special culture medium containing 1 mg/ml G418, followed by 5 days of selection in medium containing 0.4 mg/ml G418. After selection, the gene-transduced lymphocytes were expanded in round-bottom 96-well microtiter plates (Greiner Labor Technik, Nurtingen, Germany) at 37°C in 5% CO_2 in the presence of feeder cells: irradiated (25 Gy) allogeneic PBL and EBV-transformed lymphoblastoid B cell lines as described previously (29, 30). Cloning of gene-transduced T lymphocytes was accomplished through limiting dilution by seeding these T lymphocytes in round-bottom 96-well microtiter plates at 10, 3, 1, and 0.3 cells/well in the presence of feeder cells (30). The culture medium was RPMI 1640 buffered with bicarbonate (2 g/l) and HEPES (25 mM; Life Technologies), supplemented with 10% (v/v) human plasma, 150 IU/ml rIL-2, 4 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml PHA (Murex Diagnostics, Dartford, U.K.).

PCR

DNA was isolated from 10^5 PBL by incubation of the samples in DNA lysis buffer (50 mM Tris-HCl (pH 9.0), 50 mM KCl, 7 mM $MgCl_2$, 0.5% Nonidet P-40, 0.5% Tween-20, and 60 μg/ml proteinase K) at 55°C for 60 min. Proteinase K was inactivated at 95°C. DNA from 10^4 cells was amplified using the 5′ V_K primer (CTAGTCTAGAGACATTGTGATGAC CCAG) and the 3′ γ primer (GCTGCTCGAGTCTAAAGCTACTGTG GTGG). The PCR reaction was performed in a total volume of 50 μl containing 50 mM Tris-HCl (pH 9.0), 50 mM KCl, 7 mM $MgCl_2$, 200 μM of each dNTP, 20 pmol of each primer, and 0.25 U of Super Taq (HT Biotechnologies, Cambridge, U.K.) and was covered with 50 μl of paraffin oil. The samples were amplified in 35 cycles (30 s at 95°C, 30 s at 58°C, and 1 min at 72°C) using a Trio Thermoblock (Biometra, Gottingen, Germany). Twenty microliters of each sample was analyzed by agarose gel electrophoresis. For RT-PCR, RNA was isolated from 4×10^6 PBL using the RNeasy kit (Qiagen, Hilden, Germany). Single strand cDNA was synthesized by incubation of RNA with 500 ng of oligo(dt) primer (Promega, Leiden, The Netherlands) for 10 min at 75°C, followed by incubation at 37°C for 60 min with 100 U of Superscript (Life Technologies), 50 mM Tris-HCl, pH 8.3, 40 mM KCl, 6 mM $MgCl_2$, 10 mM DTT (Life Technologies), 500 μM dNTP, and 13 U RNAguard (Pharmacia, Uppsala, Sweden). PCR was subsequently performed on DNA from 10^5 cells, as described above. As a control for cDNA synthesis, amplification with β_2m primers was performed. The sequences of the β_2m primers are: β_2m 5′, TCAGGTTTACTCACGTCATCCAG; and β_2m 3′, TCACTCAATC CAAATGCGGC.

Measurement of cytokine production

To determine cytokine production by gene-transduced PBL upon Ag stimulation, 6×10^4 transduced PBL were cultured for 24 h in either the presence or the absence of 2×10^4 adherent tumor cells in RPMI culture medium containing 360 IU/ml rIL-2. Plates were centrifuged for 5 min at 1500 rpm, supernatant was harvested, and levels of TNF-α and GM-CSF were measured by ELISA (Medgenix Diagnostics, Fleuris, Belgium) according to the suppliers' specifications.

Cytotoxicity assay

Cytotoxic activity was measured in a 4- to 5-h ^{51}Cr release assay. Briefly, varying numbers of effector cells were added in triplicate to 96-well round-bottom microtiter plates (100 μl/well), followed by the addition of 2500 target cells (100 μl)/well. The target cells were labeled with 100 μCi ^{51}Cr/0.5×10^6 cells for 2 h at 37°C. At the end of the 4- to 5-h incubation period (37°C, 5% CO_2), supernatants were collected using a Skatron harvesting system (Skatron, Lier, Norway), and radioactivity was counted in a gamma counter. The percent specific lysis was calculated as follows: [(test counts − spontaneous counts)/(maximum counts − spontaneous counts)] × 100%. In blocking experiments, G250 mAb (10 μg/ml) or W6/32 mAb (10 μg/ml) was added to labeled target cells 15 to 30 min before addition of the effector cells.

CTL-target cell interactions

To examine the lytic recycling capacity of scFv/γ-transduced CTL, a secondary cytotoxicity assay that we previously developed was used (10). In short, CTL (3×10^5 or 1×10^5) were incubated for 18 h in the presence or the absence of adherent target cells (3×10^5) in 1 ml of medium supplemented with 360 IU/ml rIL-2. After exposure, transduced PBL were harvested, counted, and subsequently tested for lytic activity in the ^{51}Cr release assay as described above. Target cell contamination was determined by phase contrast microscopy.

Results

Cytokine secretion by G250 scFv/γ-transduced T lymphocytes upon stimulation with renal cell carcinoma

For expression of scFv/γ receptors selective for renal cell carcinoma we constructed one continuous molecule comprising gene

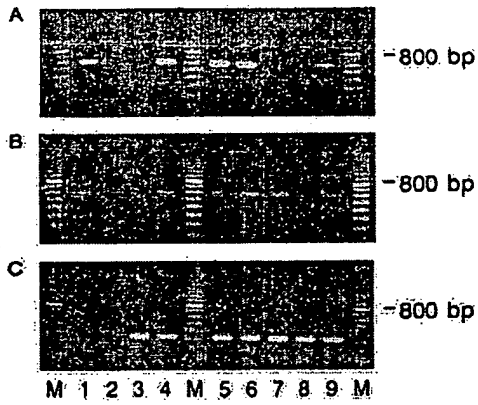

FIGURE 1. PCR analysis of the detection of scFv/γ G250 DNA and RNA in transduced T lymphocytes derived from different donors. Ethidium bromide-stained gel of DNA-PCR amplification products obtained using primers specific for scFv/γ G250 (*A*), RT-PCR amplification products using primers specific for scFv/γ G250 (*B*), and RT-PCR amplification products using β_2m primers (*C*). *Lane 1*, Positive control for scFv/γ G250-specific primers: retroviral vector containing the scFv/γ-G250 construct; *2*, negative control (no DNA/RNA control); *3*, mock-transduced PBL (PBL-LXSN); *4*, scFv/γ-transduced PBL (PBL-scG250); *5*, scFv/γ-transduced T lymphocytes derived from different donors: VD-2; *6*, VD-3; *7*, VD-4; *8*, VD-5; and *9*, VD-9. The marker (M) is a 100-bp ladder.

segments of the variable region of the renal cell carcinoma-selective mouse mAb G250 and the signal transducing human Fc(ε)RI γ-chain transmembrane and intracellular region. Retroviral gene transfer using the LXSN vector was used to generate stable integration into the genome, as we and others have previously shown to be effective in human activated T lymphocytes (31, 32). Gene transduction of activated human PBL was accomplished after cocultivation of anti-CD3-activated human PBL (frozen/thawed) with irradiated virus-producing packaging cells followed by selection in G418-containing medium and expansion in the presence of feeder cells for 2 wk. The presence of the scFv/γ viral construct in the genomic DNA of scFv/γ-transduced T lymphocytes and the expression of scFv/γ mRNA were demonstrated by DNA-PCR and RT-PCR using scFv/γ-specific primers (Fig. 1, *lanes 1–4*). Chimeric receptor surface expression was too low to be detected by FACS with anti-Id mAb (33); therefore, the percentage of scFv/γ-transduced T lymphocytes could not be determined. To examine functional expression of the receptor, the ability of scFv/γ-transduced T lymphocytes to secrete GM-CSF and TNF-α after specific target cell interaction was tested (Table I). scFv/γ-transduced bulk T lymphocytes specifically secreted GM-CSF and TNF-α after interaction with A75, a G250 mAb-binding renal cell carcinoma cell line, but not after stimulation with irrelevant SK-BR-3, a breast carcinoma cell line, thereby showing functional expression of the scFv/γ receptor and specific recognition of the relevant target cells by the scFv/γ receptor.

Cytolytic activity of G250 scFv/γ-transduced bulk CTL

To further study the functional expression of the scFv/γ receptor on transduced T lymphocytes, cytolytic activity against a panel of renal and nonrenal cell carcinoma lines was evaluated in a ^{51}Cr release assay (Fig. 2). High levels of lysis (±80% at an E:T ratio of 60) were shown against all G250 mAb-binding renal cell carcinoma cell lines. No lysis was observed of control cell lines SK-RC-59, a G250 mAb nonbinding renal cell carcinoma cell line, and G43, an irrelevant melanoma cell line (<20% at an E:T ratio of 60). In addition, no MHC-unrestricted NK activity or activated kill (AK) activity was found, as shown by the absence of lysis of K562 and Daudi, respectively (Fig. 2). That indeed the specificity of lysis was dictated by functional expression of the scFv/γ gene was further demonstrated by the inhibition of renal cell carcinoma lysis following the addition of soluble G250 mAb to the renal cell carcinoma target cells (Fig. 2). No inhibition of lysis was seen with the control anti-HLA-A,B,C Ab (W6/32; data not shown). These combined results demonstrate 1) the specificity of the G250 mAb-mediated inhibition of lysis, and 2) the non-MHC-restricted nature of the scFv/γ receptor interaction with the target cell.

Table I. *GM-CSF and TNF-α secretion of transduced PBL upon target cell interaction*[a]

	GM-CSF			TNF-α		
Effector	–	A75	SK-BR-3	–	A75	SK-BR-3
PBL-scG250	103	1326	64	<12	192	13
PBL-LXSN	99	<25	38	<12	<12	<12

[a] Six times 10^4 T lymphocytes transduced with scFv/γ (PBL-scG250) or mock-transduced (PBL-LXSN) were cultured in medium or with 2×10^4 G250 mAb-binding or non-binding tumor cells, for 24 h. Cytokine secretion in supernatant was measured by ELISA (Medgenix, Brussels, Belgium). Background cytokine secretion by tumor cells alone has been subtracted (GM-CSF: 78 pg/ml for A75 and 46 pg/ml for SK-BR-3; TNF-α: <12 pg/ml for A75 and SK-BR-3). Production of GM-CSF and TNF-α is in pg/ml/3 × 10^3 cells/24 h. A75 is a G250 binding human renal cell carcinoma cell line. SK-BR-3 is a human breast carcinoma cell line used as G250 mAb nonbinding control cell line. Similar results were obtained from 3 independent experiments. –, Indicates no stimulator cells.

Retroviral transduction has been shown to introduce foreign genes stably into lymphocytes without adversely affecting their functions over time (32). Here, we demonstrate stable functional expression over a test period of >4.5 mo of continuous culture by serial cytotoxicity studies. During this culture period we repeatedly observed specific lysis of renal cell carcinoma by bulk-cultured, gene-transduced CTL. This scFv-redirected CTL lysis was only inhibited by renal carcinoma-specific G250 mAb and was devoid of NK and AK activity. With time, an increase in $CD4^+$ T lymphocytes in the bulk population (>25%) was observed, paralleled by a decrease in specific lytic activity. Depletion of $CD4^+$ lymphocytes with magnetic beads completely restored lytic activity up to the level observed before the preferential outgrowth of $CD4^+$ lymphocytes (Fig. 3). This result demonstrates that CD4 lymphocytes are not lytic and that the decline in the percentage of $CD8^+$ CTL in favor of $CD4^+$ T lymphocytes accounts for the observed decrease in scFv/γ-mediated target cell lysis. $CD4^+$ lymphocytes do produce cytokines upon scFv/γ-mediated specific target cell interaction. Compared with the $CD8^+$ scFv/γ-transduced T lymphocytes, $CD4^+$ T lymphocytes produce approximately 5.5-fold the amount of GM-CSF and about 1.6-fold the amount of TNF-α (data not shown).

Cytolytic activity of G250 scFv/γ-transduced T lymphocyte clones

Limiting dilution of the CTL from the transduced bulk culture yielded CTL clones with multiple lytic activities. When cloned scFv/γ-transduced CTL were tested for 1) G250 scFv/γ-redirected lysis (target: SK-RC-7), 2) NK activity (target: K562), and 3) AK activity (target: Daudi), the following picture emerged: two clones with only scFv/γ-dictated lytic activity (clones 44 and 75); a clone with scFv/γ-dictated specificity and NK lytic activity (clone 27); two clones with scFv/γ, NK, and AK activities (clones 45 and 49); and a clone without cytolytic activity against the targets tested (clone 41; Fig. 4).

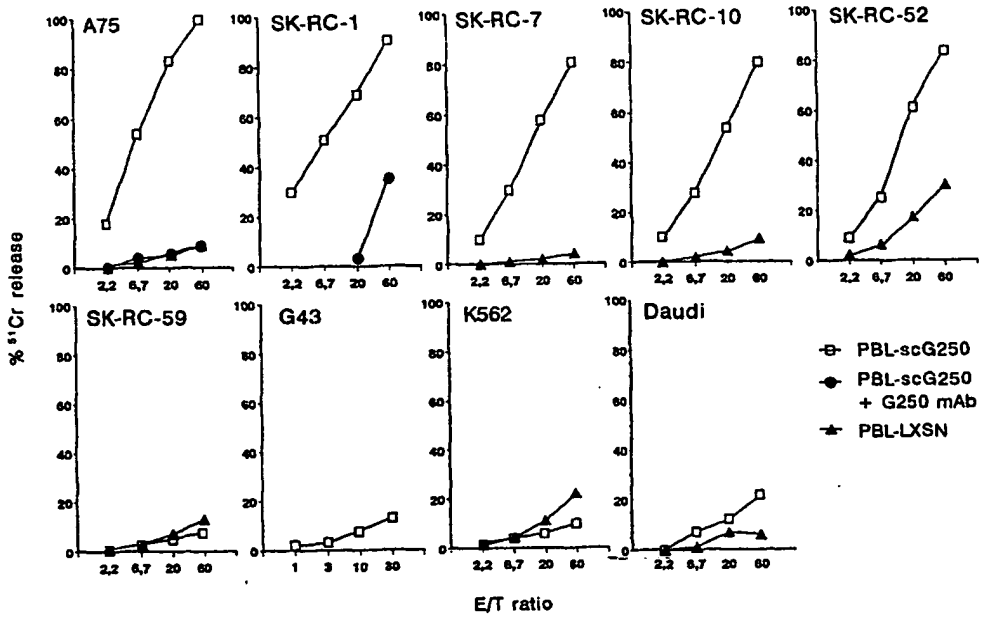

FIGURE 2. Cytotoxicity of scFv/γ (PBL-scG250) and mock (PBL-LXSN)-transduced T lymphocytes against G250 mAb-binding and nonbinding cell lines. scFv/γ-transduced (PBL-scG250) or mock-transduced (PBL-LXSN) lymphocytes were incubated with the following target cells: A75, SK-RC-1, SK-RC-7, SK-RC-10, SK-RC-52 (G250 mAb-binding renal carcinoma cell lines), SK-RC-59 (G250 mAb nonbinding renal carcinoma cell line), G43 (G250 mAb nonbinding melanoma cell line), K562, and Daudi, and tested in a 5-h cytotoxicity assay. Blocking of cytolysis with G250 mAb was performed at a concentration of 10 μg/ml. The specific ^{51}Cr release is depicted at different E:T ratios. Experiments were performed in triplicate, and the SD did not exceed 10%. Similar results were obtained from at least two independent experiments. The percentages of spontaneous release were: A75, 22%; SK-RC-1, 23%; SK-RC-7, 16%; SK-RC-10, 40%; SK-RC-52, 12%; SK-RC-59, 8%; G43, 12%; K562, 5%; Daudi, 14%.

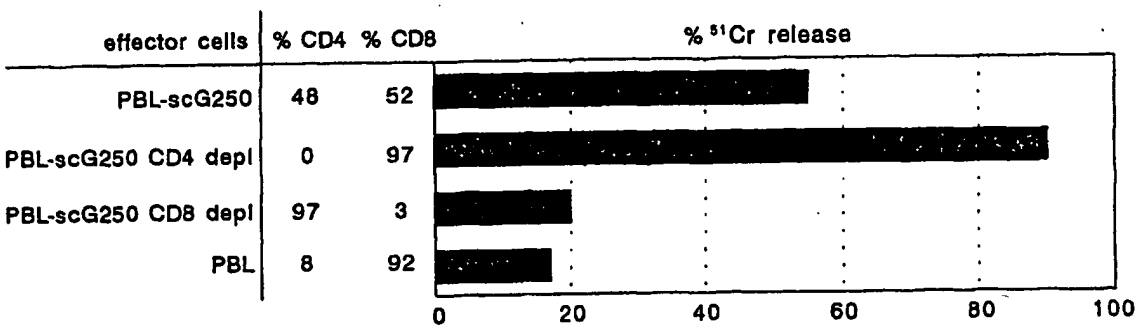

FIGURE 3. Cytotoxicity of scFv/γ (PBL-scG250), CD4-depleted fraction (PBL-scG250 CD4 depl), CD8-depleted fraction (PBL-scG250 CD8 depl), and untransduced PBL against G250 mAb-binding renal cell carcinoma, A75. CD4- and CD8-depleted PBL-scG250 fractions were obtained by magnetic bead depletion of either population of the bulk culture. The percentage of $CD4^+$ or $CD8^+$ T lymphocytes was determined by flow cytometry. Specific ^{51}Cr release is depicted at an E:T ratio of 60. Experiments were performed in triplicate, and the SD did not exceed 10%. Similar results were obtained from at least two independent experiments. The percentage of spontaneous release was 9%.

These differences in target cell specificities among CTL clones and hence CTL target recognition structures were also illustrated by the fact that only lytic activities of those CTL clones that exclusively lysed renal cell carcinoma (scFv/γ dictated) were completely inhibited by G250 mAb. The cytolytic activity of cloned scFv/γ-redirected CTL that also exerted NK and/or AK activities was partly inhibited (Fig. 4). Clones 27, 41, 45, and 49 were phenotyped and found to be $TCR\alpha\beta^+$ and $CD8^+$. Two clones (no. 41 and 49) were further analyzed and were negative for CD16, and ±50% of the T lymphocytes expressed CD56. In addition, no scFv/γ G250 membrane expression could be detected by FACS using anti-Id mAb (33) (data not shown).

Efficacy of functional LXSN scFv/γ gene transduction protocol

To investigate whether scFv/γ transduction and subsequent functional expression by recipient T lymphocytes is donor independent, we set out to transduce fresh T lymphocytes from 10 healthy volunteers. Activated PBL were cocultivated and selected as described. Evaluation of genomic DNA and RNA by PCR analysis showed T lymphocytes from all donors to contain and to transcribe the fusion gene (Fig. 1, *lanes 5–9*). Subsequent analysis of functional expression demonstrated a complete correlation between the presence of scFv/γ transcripts and 1) GM-CSF and TNF-α production upon Ag stimulation

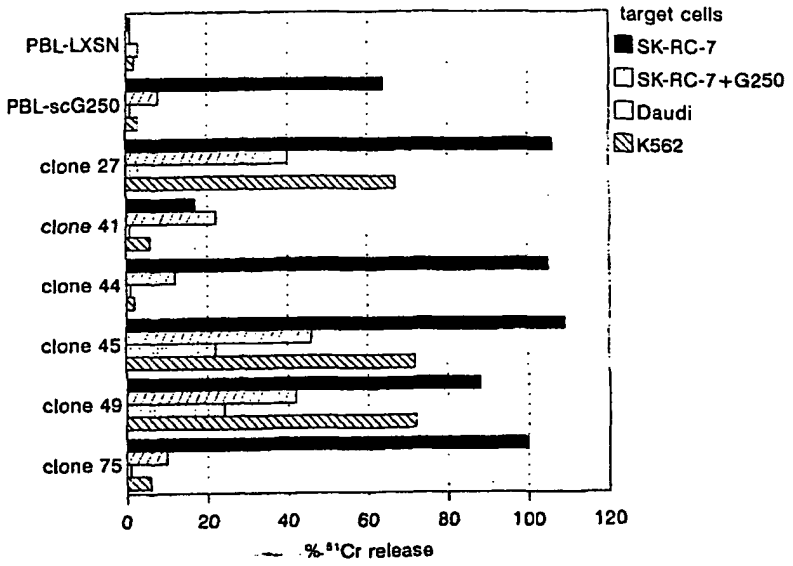

FIGURE 4. Cytotoxicity of T lymphocyte clones obtained by limiting dilution of a scFv/γ-transduced PBL population. T lymphocyte clones were tested in a 5-h ^{51}Cr release assay with target cells: K562, Daudi, or the renal carcinoma cell line SK-RC-7. Blocking of cytolysis was achieved with G250 mAb at a concentration of 10 μg/ml. The E:T ratio was 20. PBL-scG250 is the scFv/γ-transduced bulk population, and PBL-LXSN is the mock-transduced population. Experiments were performed in triplicate, and the SD did not exceed 15%. Similar results were obtained from at least two independent experiments. The percentages of spontaneous release were: SK-RC-7, 13%; Daudi, 17%; and K562, 9%.

Table II. *GM-CSF and TNF-α secretion of transduced PBL upon target cell interaction*[a]

	GM-CSF			TNF-α		
effector	–	A75	IGROV-1	–	A75	IGROV-1
VD-1	<12	716	<12	16	509	66
VD-2	<12	1500	<12	<12	1131	66
VD-3	<12	2614	<12	<12	940	52
VD-4	232	445	96	54	46	19
VD-5[b]	<12	153	<12	36	85	28
VD-6	<12	2709	<12	16	1260	56
VD-7	<12	329	<12	<12	127	16
VD-8	<12	902	<12	24	516	58
VD-9	<12	743	<12	<12	1800	<12
VD-10	<12	691	<12	<12	438	<12
PBL-LXSN	<12	31	<12	<12	<12	<12

[a] Six times 10^4 T lymphocytes transduced with scFv/γ (VD-1-10) or mock-transduced (PBL-LXSN) were cultured in medium or with 2×10^4 G250 mAb-binding or nonbinding tumor cells, for 24 h. Cytokine secretion in supernatant was measured by ELISA (Medgenix, Brussels, Belgium). Background cytokine secretion by tumor cells alone has been subtracted (GM-CSF: 206 pg/ml for A75, <12 pg/ml for IGROV-1; TNF-α: 22 pg/ml for A75, 47 pg/ml for IGROV-1). Production of GM-CSF and TNF-α is in pg/ml/3 × 10^5 cells/24 h. A75 is a G250 binding human renal cell carcinoma cell line. IGROV-1 is a human ovarium carcinoma cell line used as G250 mAb non-binding control cell line. Similar results were obtained from two independent experiments. – = no stimulator cells.

[b] GM-CSF and TNF-α in pg/ml/5 × 10^5 cells/24 h.

(Table II) or 2) scFv/γ-mediated tumor target cell lysis by the $CD8^+$-transduced CTL (Fig. 5). Cytokine production was observed in all 10 gene-transduced T lymphocyte cultures. Specific lysis of renal cell carcinoma was shown, with 8 of 10 scFv/γ-transduced T lymphocyte cultures derived from 10 donors. Specificity was further demonstrated by inhibition of renal cell carcinoma lysis by soluble parental G250 mAb to the target cells before addition of the scFv/γ-expressing CTL. To exclude the possibility that lysis might involve HLA molecules on the target cells, lysis of renal cell carcinoma by these transduced T lymphocytes derived from five different donors was also tested by preincubation of the target cell with anti-HLA framework Ab W6/32. No inhibition of lysis was seen.

As cytokine production is another sensitive method to measure specific CTL/target cell interactions, we assume that the percentages scFv/γ-transduced T lymphocytes in the two nonlytic cultures are too low to monitor lysis. Differential efficacies of gene transduction and subsequent selection of gene-transduced lymphocytes will result in T lymphocyte bulk populations comprising different percentages of gene-transduced T lymphocytes. In addition, T lymphocyte composition will vary among cultures of individual donors. This may explain the different levels of cytolytic activity and the different lytic activities (e.g., scFv/γ-dictated lysis and NK/AK activity) as well as differences in the lymphokine repertoire.

G250 scFv/γ-transduced T lymphocytes recycle chimeric receptor-dictated lytic activity

To examine the capacity of gene-transduced CTL to recycle their scFv/γ-redirected cytolysis, these CTL were allowed to enter multiple lytic interactions with relevant target cells before being tested in a secondary ^{51}Cr release assay (10). scFv/γ-transduced CTL were added to an equal (1/1) or excess (1/3) number of unlabeled G250 mAb-binding renal cell carcinoma A75 cells and incubated for 18 h. During the incubation period >95% and 60% of the target cells were lysed, respectively, as determined under the phase contrast microscope. Unlabeled irrelevant ovarian carcinoma cells (IGROV-1) were used as control target cells and were not lysed by the effector cells. scFv/γ-transduced CTL were harvested and tested for lytic activity in a secondary cytotoxicity assay in the presence of ^{51}Cr-labeled A75 cells. These preincubated cells still displayed scFv/γ-dictated cytolytic activity, which suggests that these cells, although we cannot formally exclude recruitment, retain full lytic recycling capacity (Fig. 6).

Discussion

In this report we have demonstrated redirection of human T lymphocyte specificity by retroviral single chain Ig/γ gene transfer, obtaining CTL with a permanent, MHC-unrestricted, tumoricidal activity. Redirection of lymphocyte specificity to recognize targets not recognized by their endogenous TCR was previously achieved with bsmAb, one arm of which is directed against a tumor Ag and the other against an activation molecule on T lymphocytes (4, 5). Such bsmAb-redirected CTL have shown in vivo anti-cancer activity in mice (7, 8) and in man (6, 34, 35). Lysis of tumor cells can be attributed to bsmAb-redirected $CD8^+$ T lymphocytes, although in mice additional anti-tumor growth effects were seen due to cytokine release by $CD4^+$ T lymphocytes and subsequent activation

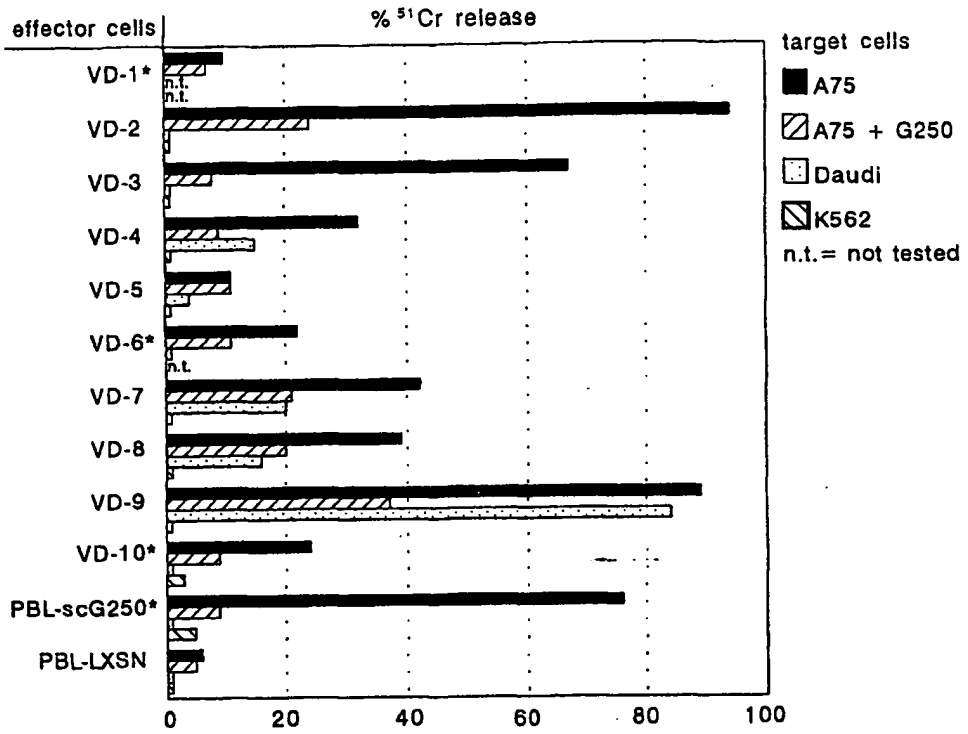

FIGURE 5. Cytotoxicity of scFv/γ-transduced CTL derived from 11 different healthy donors (VD-1–10 and PBL-scG250) and mock-transduced (PBL-LXSN) PBL against G250 mAb-binding renal carcinoma cells, A75. Gene-transduced T lymphocytes were depleted for $CD4^+$ T lymphocytes and incubated with A75, K562, or Daudi as target cells in a 4- to 5-h cytotoxicity assay. Blocking of cytolysis was achieved with G250 mAb at a concentration of 10 μg/ml. The specific ^{51}Cr release is depicted at an E:T ratio of 60 or 20 (*). Experiments were performed in triplicate, and the SD did not exceed 15%. The percentages of spontaneous release were: A75, 13%; Daudi, 6%; and K562, 5%.

of CTL and NK activities (36). Loco-regional immunotherapy in clinical studies have shown tumor responses that were restricted to the treated area (6, 35). This may be due to the limited migration capacity of bsmAb-redirected CTL and their limited recycling of signal transducing capacity, as we reported previously (10).

Genetic engineering of T lymphocytes using a scFv/γ gene carrying LXSN retroviral vector resulted in permanently acquired, Ab-dictated target cell specificity and lysis as well as the triggering of lymphokine production and of MHC-unrestricted NK/AK lysis. Such scFv/γ-redirected CTL show normal recycling of lytic activity and may have normal migration and target cell homing capacities. Indeed, we demonstrated that G250 scFv/γ-transduced CTL show relevant renal cell carcinoma-specific kill and prolonged functional expression of the chimeric receptor for a test period of almost 5 mo. Although transcription of the fusion gene could be demonstrated, levels of T lymphocyte surface expression were too low for detection by FACS analysis. Despite this low surface expression, the observed levels of lytic activities of scFv/γ-transduced CTL was high (e.g., 76% for clone 44 at an E:T ratio of 2) in a 5-h ^{51}Cr release assay. This high lytic activity may be explained by the high affinity of the scFv/γ chimeric receptor relative to that of TCR for the Ag on the target cells, although we expect the affinity of scFv/γ to be lower than the parental mAb. This low expression of scFv/γ receptors may prove advantageous from a clinical point of view, as it might lower the human anti-mouse Ab responses in patients after transfusion of scFv/γ-expressing CTL.

Because the number of tumor cells is usually in excess of lymphoid effector cell number, their elimination by CTL in vivo requires that individual CTL recycle their lytic machinery. We previously demonstrated that only in the presence of excess bsmAb

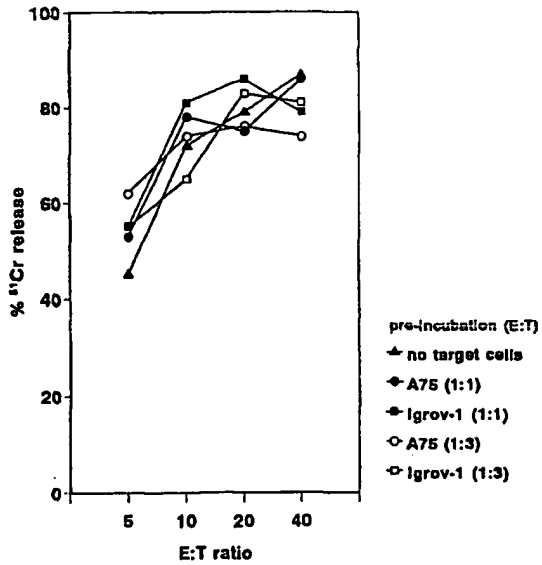

FIGURE 6. Cytolytic recycling capacity of scFv/γ-transduced PBL (PBL-scG250). Gene-transduced PBL were first exposed for 18 h to 1) medium alone, 2) G250 mAb-binding renal cell carcinoma, A75, or 3) irrelevant ovarian carcinoma cells, IGROV-1. E:T ratios were 1 and 0.3. After exposure to the target cells, retention of the cytolytic capacity of the transduced PBL was measured in a 4-h ^{51}Cr release assay against renal cell carcinoma, A75. E:T cell ratios were 5, 10, 20, and 40. Experiments were performed in triplicate, and the SD did not exceed 15%. Similar results were obtained from at least two independent experiments. The percentage of spontaneous release was 9%.

can bsmAb-redirected CTL recycle cytolytic activity (10). However, the excess bsmAb evoked a human anti-mouse Ab response, eventually blocking cytolytic activity (6, 37, 38). We, therefore, studied the lytic recycling capacity of scFv/γ-transduced CTL. Prolonged exposure (18 h) of scFv/γ-transduced CTL to their specific target cells and subsequent testing of these CTL in a secondary cytotoxicity assay demonstrated their capacity to enter multiple lytic cycles with their specific tumor targets. In normal CTL, the continued triggering of T lymphocytes and, hence, recycling of lytic capacity require continuous synthesis and subsequent surface expression of endogenous TCR (10, 39, 40). Therefore, we conclude that scFv/γ gene-transduced CTL, which functionally express the scFv/γ chimeric receptor and show recycling of lytic capacity, also continuously synthesize the transduced chimeric receptor.

Clinical application of scFv/γ-redirected PBL can be effective, as it combines induction of BRM production and cytolysis by the redirected T lymphocytes. Release of lymphokines by $CD4^+$ gene-transduced T lymphocytes upon specific target cell interaction may also contribute to tumor growth inhibition as well as induction of NK/AK activities. These lytic activities may result in elimination of those tumor cells that lack or down-regulate the relevant tumor-associated Ag expression (1).

An important feature of CTL-expressing scFv/γ receptors is that they, like Ab per se, recognize Ag in an MHC-unrestricted manner. Hence, in contrast to T lymphocytes, which recognize their ligand via their endogenous MHC-restricted TCR, the scFv/γ-dictated specificity and anti-tumor activity is not adversely affected by tumor cells that down-regulate their MHC complex or for other reasons do not express MHC-restricted Ags (41).

In conclusion, we have shown long term functional expression of scFv/γ chimeric receptors by transduced T lymphocytes, scFv/γ-triggered lymphokine production, and cytolysis of relevant target cells as well as recycling of lytic activity. The scFv/γ gene transduction was successful in all 11 donors, and these results provided "proof of principle" of the use of scFv/γ-expressing CTL for clinical anti-cancer treatment.

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References

1. Stoter, G., S. H. Goey, D. Batchelor, A. M. Eggermont, C. Lamers, J. W. Gratama, and R. L. H. Bolhuis. 1992. Treatment of disseminated renal cell cancer with combinations of interleukin-2, lymphokine-activated killer cells, and α-interferon. In *EORTC Genitourinary Group Monograph 11: Recent Progress in Bladder and Kidney Cancer.* F. H. Schröder, ed. Wiley-Liss, Inc., New York, p. 225.
2. Rosenberg, S. A., J. R. Yannnelli, J. C. Yang, S. L. Topalian, D. J. Schwartzentruber, J. S. Weber, D. R. Parkinson, C. A. Seipp, J. H. Einhorn, and D. E. White. 1994. Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2. *J. Natl. Cancer Inst. 86:1159.*
3. Weidmann, E., M. Trucco, and T. L. Whiteside. 1994. Relevance of the T cell receptor for immunotherapy of cancer. *Cancer Immunol. Immunother. 39:1.*
4. Lanzavecchia, A., and D. Scheidegger. 1987. The use of hybrid hybridomas to target human cytotoxic T lymphocytes. *Eur. J. Immunol. 17:105.*
5. Van Dijk, J., S. O. Warnaar, J. D. H. van Eendenburg, M. Thienpont, E. Braakman, J. H. A. Boot, G. J. Fleuren, and R. L. H. Bolhuis,. 1989. Induction of tumor cell lysis by bi-specific monoclonal antibodies recognizing renal cell carcinoma and CD3 antigen. *Int. J. Cancer 43:344.*
6. Bolhuis, R. L. H., C. H. J. Lamers, S. H. Goey, A. M. M. Eggermont, J. B. M. Z. Trimbos, G. Stoter, A. Lanzavecchia, E. Di Re, S. Miotti, F. Raspagliesi, L. Rivoltini, and M. I. Colnaghi. 1992. Adoptive immunotherapy of ovarian carcinoma with bsmAb-targeted lymphocytes: a multicenter study. *Int. J. Cancer Suppl. 7:78.*
7. Renner, C., W. Jung, U. Sahin, R. Denfeld, C. Pohl, L. Trümper, F. Hartmann, V. Diehl, R. van Lier, and M. Pfreundschuh. 1994. Cure of xenografted human tumors by bispecific antibodies and human T cells. *Science 264:833.*
8. Mezzanzanica, D., M. A. Garrido, D. S. Neblock, P. E. Daddona, S. M. Andrew, V. R. Zurawski, Jr., D. M. Segal, and J. R. Wunderlich. 1991. Human T lymphocytes targeted against an established human ovarian carcinoma with a bispecific F(ab')$_2$ antibody prolong host survival in a murine xenograft model. *Cancer Res. 51:5716.*
9. Jain, R. K. 1989. Delivery of novel therapeutic agents in tumors: physiological barriers and strategies. *J. Natl. Cancer Inst. 81:570.*
10. Blank-Voorthuis, C. J. A. C., E. Braakman, C. P. M. Ronteltap, B. C. Tilly, E. Sturm, S. O. Warnaar, and R. L. H. Bolhuis. 1993. Clustered CD3/TCR complexes do not transduce activation signals after bispecific monoclonal antibody-triggered lysis by CTL via CD3. *J. Immunol. 151:2904.*
11. Eshhar, Z., T. Waks, G. Gross, and D. G. Schindler. 1993. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the γ or ζ subunits of the immunoglobulin and T-cell receptors. *Proc. Natl. Acad. Sci. USA 90:720.*
12. Stancovski, I., D. G. Schindler, T. Waks, Y. Yarden, M. Sela, and Z. Eshhar. 1993. Targeting of T lymphocytes to Neu/HER2-expressing cells using chimeric single chain Fv receptors. *J. Immunol. 151:6577.*
13. Bird, R. E., K. D. Hardman, J. W. Jacobson, S. Johnson, B. M. Kaufman, S. M. Lee, T. Lee, S. H. Pope, G. S. Riordan, and M. Whitlow. 1988. Single chain antigen-binding proteins. *Science 242:423.*
14. Colcher, D., R. Bird, M. Roselli, K. D. Hardman, S. Johnson, S. Pope, S. W. Dodd, M. W. Pantoliano, D. E. Milenic, and J. Schlom. 1990. In vivo tumor targeting of a recombinant single chain antigen-binding protein. *J. Natl. Cancer Inst. 82:1191.*
15. Wels, W., I. M. Harwerth, N. E. Hynes, and B. Groner. 1992. Diminution of antibodies directed against tumor cell surface epitopes: a single chain Fv fusion molecule specifically recognizes the extracellular domain of the c-*erb* B2 receptor. *J. Steroid Biochem. Mol. Biol. 43:1.*
16. Brocker, T., A. Peter, A. Traunecker, and K. Karjalainen. 1993. New simplified molecular design for functional T cell receptor. *Eur. J. Immunol. 23:1435.*
17. Moritz, D., W. Wels, J. Mattern, and B. Groner. 1994. Cytotoxic T lymphocytes with grafted recognition specificity for ERBB2-expressing tumor cells. *Proc. Natl. Acad. Sci. USA 91:4318.*
18. Hwu, P., G. E. Shafer, J. Treisman, D. G. Schindler, G. Gross, R. Cowherd, S. A. Rosenberg, and Z. Eshhar. 1993. Lysis of ovarian cancer cells by human lymphocytes redirected with a chimeric gene composed of an antibody variable region and the Fc receptor γ chain. *J. Exp. Med. 178:361.*
19. Roberts, M. R., L. Qin, D. Zhang, D. H. Smith, A.-C. Tran, T. J. Dull, J. E. Groopman, D. J. Capon, R. A. Byrn, and M. H. Finer. 1994. Targeting of human immunodeficiency virus-infected cells by $CD8^+$ T lymphocytes armed with universal T-cell receptors. *Blood 84:2878.*
20. Oosterwijk, E., D. J. Ruiter, P. J. Hoedemaker, E. K. J Pauwels, U. Jonas, J. Zwartendijk, and S. O. Warnaar. 1986. Monoclonal antibody G250 recognizes a determinant present in renal cell carcinoma and absent from normal kidney. *Int. J. Cancer 38:489.*
21. Van Dijk, J., E. Oosterwijk, M. J. P. G. van Kroonenburg, U. Jonas, G. J. Fleuren, E. K. J. Pauwels, and S. O. Warnaar. 1988. Perfusion of tumor-bearing kidneys as a model for scintigraphic screening of monoclonal antibodies. *J. Nucl. Med. 29:1078.*
22. Küster, H., H. Thompson, and J.-P. Kinet. 1990. Characterization and expression of the gene for the human Fc receptor γ subunit. *J. Biol. Chem. 265:6448.*
23. Berke, G., K. A. Sullivan, and D. B. Amos. 1972. Tumor immunity in vitro: destruction of a mouse ascites tumor through a cycling pathway. *Science 177: 433.*
24. Zagury, D., J. Bernard, N. Thierness, M. Feldman, and G. Berke. 1975. Isolation and characterization of individual functionally reactive cytotoxic T lymphocytes: conjugation, killing and recycling at the single cell level. *Eur. J. Immunol. 5:818.*
25. Lamers, C. H. J., R. J. van de Griend, E. Braakman, C. P. M. Ronteltap, J. Bénard, G. Stoter, J. W. Gratama, and R. L. H. Bolhuis. 1992. Optimization of culture conditions for activation and large-scale expansion of human T lymphocytes for bispecific antibody-directed cellular immunotherapy. *Int. J. Cancer 51:973.*
26. Loh, E. Y., J. F. Elliot, S. Cwirla, L. L. Lanier, and M. M. Davis. 1989. Polymerase chain reaction with single-sided specificity: analysis of T cell receptor δ chain. *Science 243:217.*
27. Weissman, A. M., D. Hou, D. G. Orloff, W. S. Modi, H. Seuanez, S. J. O'Brien, and R. D. Klausner. 1988. Molecular cloning and chromosomal localization of the human T-cell receptor ζ chain: distinction from the molecular CD3 complex. *Proc. Natl. Acad. Sci. USA 85:9709.*
28. Miller, A. D., and G. J. Rosman. 1989. Improved retroviral vectors for gene transfer and expression. *Biotechniques 7:980.*
29. Van de Griend, R. J., B. A. van Krimpen, S. J. L. Bol, A. Thompson, and R. L. H. Bolhuis. 1984. Rapid expansion of human cytotoxic T cell clones: growth promotion by a heat-labile serum component and by various types of feeder cells. *J. Immunol. Methods 66:285.*
30. Van de Griend, R. J., and R. L. H. Bolhuis. 1984. Rapid expansion of allospecific cytotoxic T cell clones using nonspecific feeder cell lines without further addition of exogenous IL-2. *Transplantation 38:401.*
31. Kasid, A., S. Morecki, P. Aebersold, K. Cornetta, K. Culver, S. Freeman, E. Director, M. T. Lotze, R. M. Blease, W. F. Anderson, and S. A. Rosenberg. 1990. Human gene transfer: characterization of human tumor-infiltrating lymphocytes as vehicles for retroviral-mediated gene transfer in man. *Proc. Natl. Acad. Sci. USA 87:473.*
32. Braakman, E., V. W. van Beusechem, B. A. van Krimpen, A. Fisher, R. L. H. Bolhuis, and D. Valerio. 1992. Genetic correction of cultured T cells from an adenosine deaminase-deficient patient: characteristics of non-transduced and transduced T cells. *Eur. J. Immunol. 22:63.*
33. Uemura, H., E. Okajima, F. M. J. Debruyne, and E. Oosterwijk. 1994. Internal image anti-idiotype antibodies related to renal cell carcinoma-associated antigen G250. *Int. J. Cancer 56:609.*

34. Nitta, T., K. Sato, H. Yagita, K. Okumura, and S. Ishii. 1990. Preliminary trial of specific targeting therapy against malignant melanoma. *Lancet 335:368.*
35. Canevari, S., G. Stoter, F. Arienti, G. Bolis, M. I. Colnaghi, E. M. Di Re, A. M. M. Eggermont, S. H. Goey, J. W. Gratama, C. H. J. Lamers, M. A. Nooy, G. Parmiani, F. Raspagliesi, F. Ravagnani, G. Scarfone, J. B. Trimbos, S. O. Warnaar, and R. L. H. Bolhuis. 1995. Regression of advanced ovarian carcinoma by intraperitoneal treatment with autologous T lymphocytes retargeted by a bispecific monoclonal antibody: clinical proof of concept. *J. Natl. Cancer Inst. 87:1463.*
36. Qian, J.-H., J. A. Titus, S. M. Andrew, D. Mezzanzanica, M. A. Garrido, J. R. Wunderlich, and D. M. Segal. 1991. Human peripheral blood lymphocytes targeted with bispecific antibodies release cytokines that are essential for inhibiting tumor growth. *J. Immunol. 146:3250.*
37. Lind, P., P. Lechner, B. Hausmann, M. G. Smola, P. Koeltringee, P. Steindorfer, H. Cesnik, R. Passl, and O. Eber. 1991. Development of human anti-mouse antibodies (HAMA) after single and repeated diagnostic application of intact murine monoclonal antibodies. *Antib. Immunoconjug. Radiopharm. 4:811.*
38. Lamers, C. H. J., J. W. Gratama, S. O. Warnaar, G. Stoter, and R. L. H. Bolhuis. 1995. Inhibition of bispecific monoclonal antibody (bsAb)-targeted cytolysis by human anti-mouse antibodies in ovarian carcinoma patients treated with bsAb-targeted activated T lymphocytes. *Int. J. Cancer 60:450.*
39. Reinherz, E. L., S. Meuer, K. A. Fitzgerald, R. E. Hussey, H. Levine, and S. F. Schlossman. 1982. Antigen recognition by human T lymphocytes is linked to surface expression of the T3 molecular complex. *Cell 30:735.*
40. Valitutti, S., S. Müller, M. Cella, E. Padovan, and A. Lanzavecchia. 1995. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature 375:148.*
41. Refisto, N. P., Y. Kawakami, F. Marincola, P. Shamamian, A. Taggarse, F. Esquivel, and S. A. Rosenberg. 1993. Molecular mechanisms used by tumors to escape immune recognition: immunogenethempy and the cell biology of major histocompatibility complex class I. *J. Immunother. 14:182.*

1: Gene Ther. 1998 Sep;5(9):1195-203.

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A retroviral vector system 'STITCH' in combination with an optimized single chain antibody chimeric receptor gene structure allows efficient gene transduction and expression in human T lymphocytes.

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Genetic engineering of T lymphocytes for adoptive clinical immunotherapy calls for efficient gene transduction methods. Therefore, a transient retroviral gene transduction system 'STITCH' was developed comprising pSTITCH retroviral vector encoding the transgene, plasmids encoding Moloney murine leukemia virus gag/pol and gibbon ape leukemia virus envelope, and the human kidney cell line 293T as a packaging line. Cotransfection of retroviral vector and packaging plasmids in 293T cells results in the production of GALV env pseudotyped viral particles with a titer of 10(7) infectious units per milliliter. The 'STITCH' gene transduction system efficiently transduces genes into activated human T lymphocytes derived from healthy donors and cancer patients. The efficacy of gene transduction is donor-independent. A direct application of the 'STITCH' gene transduction system is the genetic engineering of activated human T lymphocytes to induce expression of antibody based chimeric receptors in their membrane. Introduction of these chimeric receptors into activated human T lymphocytes graft these cells with specificity for, for example, renal cell carcinoma. In order to study the effect of the chimeric receptor gene structure on the processes ultimately leading to functional membrane expression, we designed a number of different chimeric receptor gene structures and subsequently compared their membrane expression on 293T cells and activated human T lymphocytes. Distinct membrane expression densities were observed on 293T cells and human T lymphocytes for the different chimeric receptor gene constructs. Gene transduction of activated human T lymphocytes with four out of five chimeric receptor gene constructs resulted in functional expression of chimeric receptor as demonstrated by specific recognition and cytolysis of renal cell carcinoma.

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Chimeric scFv/gamma receptor-mediated T-cell lysis of tumor cells is coregulated by adhesion and accessory molecules.

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Adhesion and accessory molecules play a critical role in T-cell activation and effector function in general and in tumor cell recognition and lysis in particular. We investigated the contribution of CD2, CD3, CD11a/CD18, CD54 and CD58 molecules in T lymphocyte-tumor cell interactions mediated by chimeric immunoglobulin receptors. The chimeric receptor is composed of a single chain antibody binding site and a gamma-chain signal transducing molecule (scFv/gamma). T lymphocytes expressing such scFv/gamma receptors recognize the G250 Ag on renal cell carcinoma (RCC) in an major histocompatibility complex (MHC)-unrestricted manner and exert RCC selective cytolysis. A coregulatory role for CD2, CD3 and CD11a/CD18 molecules in scFv/gamma-mediated cytolysis was demonstrated using monoclonal antibody (MAb)-induced inhibition of scFv/gamma-mediated cytolysis. The inhibition of lysis was not due to inhibition of cytotoxic T lymphocyte (CTL)-target cell conjugation but rather to a post-conjugate signaling event. Binding of CD54 and CD58 MAbs to the RCC did not inhibit cytolysis of RCC cells that expressed high levels of both CD54 and the G250 antigen (Ag) (A75), whereas cytolysis of RCC expressing intermediate levels of CD54 and G250 Ag (SK-RC-17 cl.4) was partly inhibited by the CD54 MAb. Binding of low concentrations of G250 MAb to RCC (A75) rendered these cells sensitive to CD54 MAb inhibition, demonstrating a direct functional relation between G250 Ag expression level and adhesion molecules. Taken together, our findings indicate a coregulatory role for CD2, CD3 and CD11a/CD18 molecules in the scFv/gamma-mediated cytolysis of tumor cells and show that the requirement of CD11a/CD18-CD54 interactions is dependent on the level of free Ag. This make these gene-transduced T lymphocytes attractive tools for adoptive immunogene therapy of cancer.

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